

**Developing new models to predict treatment outcome in patients with
chronic hepatitis C infection**

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Abstract

Chronic infection with hepatitis C virus (HCV) is a significant global health problem, with approximately 170 million individuals infected worldwide. Treatment is rapidly evolving to combinations of highly potent direct-acting antiviral drugs, although optimal combinations for individual patient groups are not yet clear. Treatment failure still occurs, and relapse may occur in as many as 10% of treated individuals.

Whilst HCV replicates primarily in the liver, HCV RNA has been identified in extrahepatic sites, including monocytes, although extrahepatic replication is controversial. Our group recently developed a novel assay to study replication of patient-derived HCV by fusion of patient monocytes with cultured hepatoma cells. In this study, we extended the utility of this fusion model, by using cell culture monocytes to 'capture' HCV from patient serum prior to fusion with hepatoma cells. We studied whether this 'capture-fusion' model could be used to screen individual patient drug response and the mechanisms permitting viral replication in the hybrid cells.

Using the capture-fusion assay, patient-derived HCV of all viral genotypes replicated to levels detectable by a sensitive PCR assay and could be inhibited by antiviral drugs in a genotype-specific manner. Isolates with genotypic and phenotypic drug resistance could be identified, and, in a blinded study, drug sensitivity in the assay correlated with clinical treatment response. Relapse after interferon and ribavirin treatment was associated with poor pre-treatment ribavirin sensitivity and also with viable HCV in patient-derived monocytes at the end of therapy. Uptake of HCV into monocytes appeared independent of classical HCV entry receptors, and our work implicated CD64 in HCV monocyte entry. Cell fusion appeared to permit transfer of HCV RNA to hepatocytes without triggering an intracellular antiviral innate immune response.

Our data suggest that this novel 'capture-fusion' model represents a promising new technique which may help identify appropriate treatment strategies for patients with chronic HCV infection.

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List of commonly used abbreviations

ALT	alanine aminotransferase
ATP	adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary DNA
CLDN1	claudin-1
CNS	central nervous system
CV	coefficient of variation
DAA	direct-acting antiviral agents
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DVR	delayed virological response
EDTA	ethylenediaminetetraacetic acid
EVR	early virological response
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
G	(HCV) genotype
HCV	Hepatitis C virus
HCVcc	cell culture produced HCV
HCVpp	HCV pseudoparticles
HDL	high density lipoprotein
IC ₅₀	50% inhibitory concentration
IFN α	interferon- α
IFN γ	interferon- γ
ISDR	interferon sensitivity determining region
IRES	internal ribosomal entry site
ISG	interferon stimuable gene
IU	international units
JFH-1	Japanese fulminant hepatitis-1
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor

LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
NI	nucleoside inhibitor
NNI	non-nucleoside inhibitor
NR	non-response (to antiviral therapy)
NS	non-structural protein
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pegIFN	pegylated interferon
PFA	paraformaldehyde
PHH	primary human hepatocytes
PI	protease inhibitor
PMA	phorbol 12-myristate 13-acetate
RAV	resistance associated variant
RBV	ribavirin
RIG-1	retinoic acid inducible gene-1
RNA	ribonucleic acid
RT	reverse transcription
RT-qPCR	one-step reverse transcription-quantitative polymerase chain reaction
RVR	rapid virological response
Sd	standard deviation
SEAP	secreted alkaline phosphatase
Sem	standard error of the mean
SNP	single nucleotide polymorphism
SR-B1	scavenger receptor B-1
ssRNA	single stranded RNA
SVR	sustained virological response
TCID ₅₀	50% tissue culture infectious dose
UTR	untranslated region
VLDL	very low density lipoprotein

1.0 Introduction

1.1 Clinical impact of HCV infection

Hepatitis C virus (HCV) infection represents a significant global health problem, with 170 million individuals estimated to be chronically infected worldwide [1]. Only a minority of those exposed to the virus clear it spontaneously, with chronic HCV developing in around 80% of those infected. Chronic HCV infection frequently leads to development of liver fibrosis, progressing to cirrhosis in 20-30% of those infected for 20 years [2]. Acute infection is usually asymptomatic and chronically infected individuals often remain unaware of the diagnosis until advanced liver disease has developed. Once cirrhosis is established, there is a risk of hepatocellular carcinoma (HCC) of approximately 4% per annum, and of decompensation, death or need for liver transplantation of around 6% per annum [3]. In the UK, deaths due to HCV related liver disease continue to rise, and based on current levels of diagnosis and treatment, are predicted to continue to do so until at least 2020 [4]. Successful treatment with eradication of the virus can halt the progression of liver fibrosis and significantly reduce development of complications in patients with cirrhosis [5].

1.1.1 Genotypic distribution of HCV

HCV is subdivided into seven major genotypes, which vary by up to 30% at the nucleotide level, and numerous subtypes which vary by at least 15% [6, 7]. Viral genotypes reflect global diversity and evolution of the virus, and therefore show a geographical distribution. Genotype (G)1 is most commonly found in Western Europe, North America and Japan. G2 is common in Europe, whilst G3 predominates in South East Asia. G4 infection is seen predominantly in North

Africa, G5 in South Africa, G6 in the Far East, particularly Vietnam, and G7 has been isolated from a single emigrant from the Congo [7]. In the UK, the genotypic burden of HCV infection is 45% G1, 44% G3, 7% G2 and 3% G4, with the remainder comprising G5 or 6, or unclassifiable strains [8]. Knowledge of the infecting genotype is important as it influences treatment response. Whether it affects disease progression has been controversial, but there is some evidence to suggest that G3 infection is associated with faster progression of fibrosis than other viral genotypes [9].

1.2 The hepatitis C virus

1.2.1 Taxonomy and genome organisation

HCV is the sole member of the *Hepacivirus* genus of the *Flaviviridae* family, which also includes dengue virus and yellow fever (*Flavivirus* genus). Its 9.6kb positive-strand RNA genome comprises a highly conserved 5' untranslated region containing the internal ribosomal entry site, an open reading frame which encodes ten viral structural and non-structural proteins, and a 3' non-coding region (Figure 1-1). The structural proteins consist of the envelope proteins (E1 and E2) and the core protein. The non-structural proteins comprise the p7 ion channel, NS2-3 protease, NS3 serine protease and RNA helicase, the NS4A polypeptide, NS4B, NS5A which forms a critical component of the viral replication complex, and NS5B which is the viral RNA-dependent RNA polymerase (RdRp).

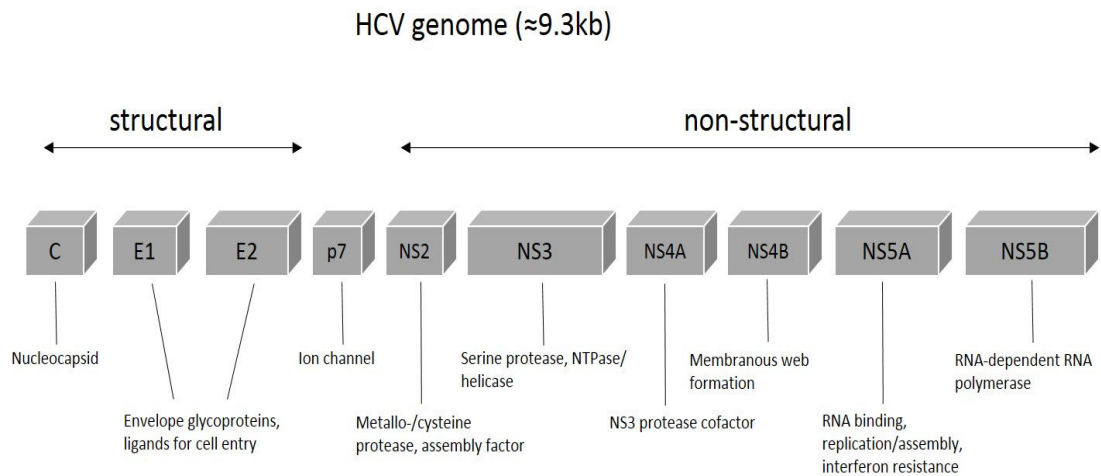


Figure 1-1. Organisation of the HCV genome. Adapted from Lange and Sarrazin (2014) [10].

1.2.2 HCV cell entry

HCV cell entry is a complex, multi-step process which remains incompletely understood. A number of the receptors and co-factors implicated in the process have recently been identified, and much of the current understanding of HCV cell entry is summarised in Figure 1-2.

The hepatocyte tropism of HCV may be at least partially determined by entry receptor expression. All of the proteins implicated in HCV cell entry are expressed on hepatocytes; these include the tetraspanin CD81, scavenger receptor B1 (SR-B1), the tight junction proteins claudin-1 (CLDN1) and occludin and the low density lipoprotein receptor (LDLR). Other receptors or co-factors may also play a role, including glycosaminoglycans (GAGs), the epidermal growth factor receptor (EGFR) and the cholesterol receptor NPC1L1.

Glycosaminoglycans have been implicated in early non-specific attachment of HCV particles, and may serve to concentrate virions on the surface of the host cell to facilitate downstream interaction with specific receptors [11, 12]. HCV circulates in the blood in a number of forms, which can be separated according to buoyant density. The virions with high buoyant density appear to be associated with immunoglobulin [13], whilst those in the low density fraction are associated with low-density and very low-density lipoproteins [14, 15]. Virions found in the lower buoyant density fraction of plasma are more infectious than those with higher buoyant density [16], implying that association with low density lipoprotein may enhance infectivity [17]. The low density lipoprotein receptor (LDLR) has been implicated as a candidate HCV receptor. Endocytosis of HCV from patient serum into immortalised B cells or hepatoma cells was increased by upregulation of the LDLR, and decreased in a dose-dependent manner by inhibition with an anti-LDLR antibody [18]. Subsequent studies have supported a role for LDLR in entry of serum-derived HCV into primary human hepatocytes [19], and suggested an interaction between LDLR and apolipoprotein E present in the lipoviral particle [20]. However a recent report suggests that the LDLR entry pathway may not lead to productive infection, but rather a functional LDLR may be required for viral replication [21]. Thus while the LDLR certainly appears to be important in the HCV life cycle, its precise role remains to be fully elucidated.

SR-B1 is an integral membrane protein found mainly in the liver, adrenal and intestine. In the liver, it facilitates uptake of cholesterol esters from high density lipoprotein (HDL) as part of cholesterol homeostasis. SR-B1 overexpression increases HCV infection, whilst blocking antibodies against SR-B1 reduce

infectivity of human hepatoma cells [22]. However, it has also been observed that HDL increases, whilst oxidised LDL inhibits HCV infectivity and these effects are dependent on SR-B1 [23]. This suggests that the interaction between SR-B1 and HCV is more complex than simple virus-receptor binding. A number of reports suggest that in addition to its role in lipid homeostasis SR-B1 can modulate plasma membrane composition [24]. Although the exact role of SR-B1 in HCV cell entry has yet to be described, an early HCV interaction with SR-B1 may facilitate removal of lipid from the viral particle or induce conformational change in the HCV glycoproteins which primes subsequent receptor binding. Alternatively, the HCV - SR-B1 interaction may trigger conformational changes in the plasma membrane which bring the virus into close proximity with additional entry receptors [25].

CD81 is a ubiquitously expressed transmembrane protein which interacts with HCV E2 via a long extracellular loop [26]. A number of lines of evidence indicate that CD81 is a critical entry factor for HCV. Anti-CD81 blocking antibodies can neutralise HCV infection, which is also abolished by small interfering RNA (siRNA)-mediated knockdown of CD81 expression [26-29]. Ectopic expression of CD81 in HepG2 cells, a liver cell line which does not normally express CD81 and is not permissive to HCV infection, permitted infection of these cells [26, 29, 30]. However, expression of CD81 alone is not sufficient for HCV entry [26]. Increasingly, it appears that CD81 interacts with claudin-1 (CLDN1) to permit HCV entry. CLDN1 is a member of the claudin family of tight junction proteins which is highly expressed in the liver, but also in other epithelial tissues. CLDN1 appears necessary for infection of human hepatoma cells, and ectopic

expression of CLDN-1 renders non-hepatic cells permissive to HCV infection [31-33]. It appears to play a role relatively late in viral entry, probably after viral interaction with CD81 [32]. Anti-CLDN1 antibodies which inhibit viral entry appear to act by inhibiting the interaction between CLDN1 and CD81 [34]. The natural occurrence of CLDN1-CD81 complexes in a variety of cell types has been demonstrated, and disruption of these complexes inhibits HCV entry [35, 36].

Although SR-B1, CD81 and CLDN1 are all essential for HCV entry into hepatocytes, the expression of these proteins is not sufficient to allow infection of non-permissive cells [32]. Occludin, a transmembrane protein present in the tight junction complex of polarised epithelial cells which is highly expressed in the liver, was recently identified as a fourth host cell protein required for HCV cell entry [37]. Expression of human SR-B1, CD81, CLDN1 and occludin, but not single, double or triple combinations of these receptors, renders normally non-permissive murine cells susceptible to HCV infection [37, 38]. Its precise role in HCV entry remains to be described.

Most recently, other proteins have been postulated to act as co-factors for HCV entry. The Niemann-Pick C1-like cholesterol receptor (NPC1L1) has been implicated as a co-factor acting late in the sequence of binding and internalisation [39]. Other host factors, particularly cytokines and growth factors, have been implicated in HCV cell entry. For example, stimulation of the ephrin receptor (EphAR) or epidermal growth factor receptor (EGFR) enhances HCV entry, whilst silencing or inhibition reduces entry. A role for these receptor tyrosine kinases in

the association between CD81 and CLDN1 and in membrane fusion has been suggested [40].

The current model for HCV entry into hepatocytes, summarised in Figure 1-2, suggests an initial interaction of the virus with GAGs and LDLR prior to interaction with CD-81/CLDN1 complexes. This interaction may trigger translocation of the virus-receptor complex to the intercellular tight junctions, where it interacts with occludin prior to internalisation via clathrin-mediated endocytosis [41, 42]. This is followed by pH-dependent membrane fusion within early endosomes and release of the viral genome into the cytoplasm [43].

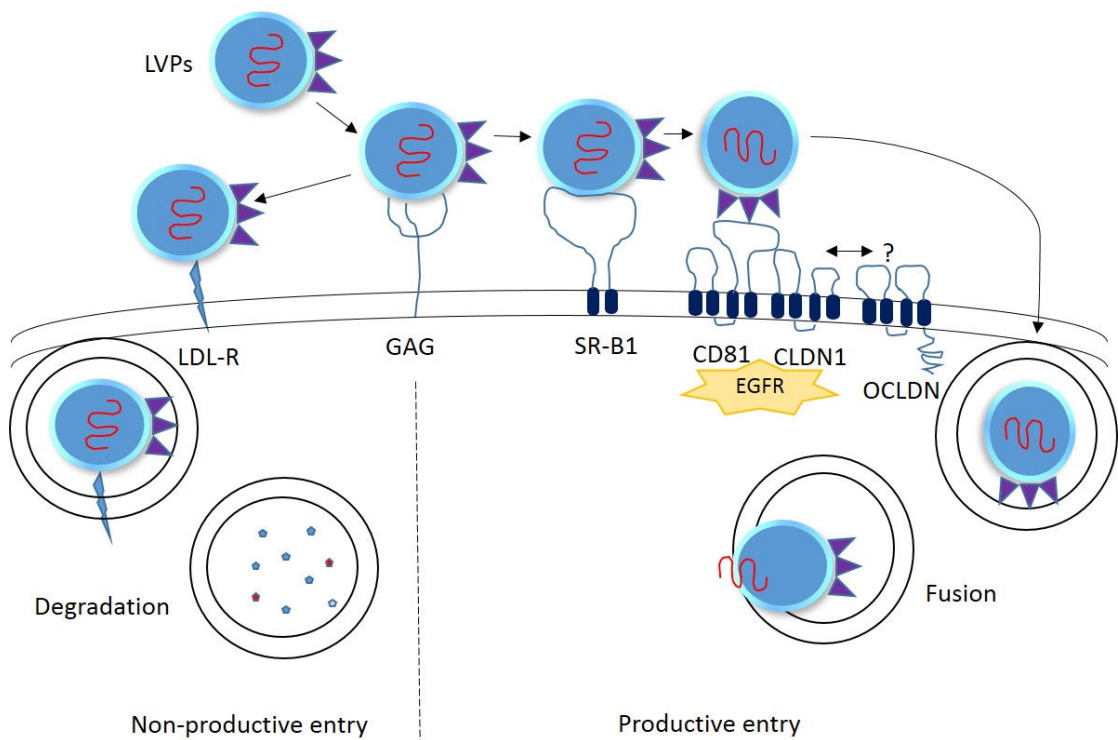


Figure 1-2. Schematic representation of current understanding of HCV entry into hepatocytes. LVPs, lipo-viral particles; LDL-R, low density lipoprotein receptor; GAG, glycosaminoglycans; SR-B1, scavenger receptor B1; CLDN1, claudin 1; OCLN, occludin. Adapted from Ploss and Dubuisson (2012) [44].

1.2.3 HCV replication cycle

To date, the events governing viral RNA translation, replication, and genome packaging for cell exit are poorly characterised. The current understanding of the HCV life cycle is summarised in Figure 1.3.

The 5' terminus of the HCV genome contains the internal ribosomal entry site (IRES) which is involved in ribosome binding and initiation of translation of the genome [45]. Translation yields a polyprotein precursor of approximately 3000 amino acids. This polyprotein is co- and post-translationally cleaved by both cellular and viral proteases to yield the mature structural and non-structural proteins [46]. The current understanding of the functions of the viral proteins is summarised in Table 1-1.

HCV Protein	Function
Core	Capsid forming protein; regulatory role in translation, RNA replication and particle assembly
Envelope glycoprotein 1 (E1)	Adsorption, receptor-mediated endocytosis
Envelope glycoprotein 2 (E2)	Adsorption, receptor-mediated endocytosis
P7	Forms an ion channel in the endoplasmic reticulum. Essential for formation of infectious virions
NS2	Component of the NS2-3 protease, which cleaves the viral polyprotein between NS2 and NS3. Role in HCV assembly.
NS3	NS2-3 protease, cleavage of downstream HCV proteins. ATPase/helicase activity, unwinding of viral RNA. Interference in innate immune sensing of HCV infection (via the NS3-4A protease, see text).
NS4A	Component of NS3-4A protease.
NS4B	Induces membranous web at the endoplasmic reticulum during HCV RNA replication.
NS5A	Multi-functional phosphoprotein implicated in formation of the HCV replication complex, virion assembly and manipulation of the host-cell environment. Contains interferon-sensitivity determining region.
NS5B	Viral RNA-dependent RNA polymerase

Table 1-1. Current understanding of the functions of the HCV proteins. Adapted from Kupfer (2012) [47] and Ross-Thriepland and Harris (2015) [48].

In common with other positive strand RNA viruses, replication of the viral genome appears to occur within a membrane-associated replication complex [49]. The viral NS4B protein induces formation of a “membranous web” consisting of vesicles in a membranous matrix, derived primarily from the endoplasmic reticulum [50]. Domain I of the non-structural protein NS5A is also implicated in formation of the membranous web [51], and biogenesis of the membranous web is blocked by NS5A inhibitors [52, 53]. All components of the HCV polyprotein associated with this membranous web when visualised by electron microscopy [49], and it was identified as the site of HCV RNA synthesis in hepatoma cell lines [54]. The reasons for assembly of the membranous web for viral replication are mainly speculative, and may include protection viral RNA/dsRNA from cellular innate immune sensing machinery or RNA interference, physical support for both the organisation of the viral replication complex and also tethering of unwinding viral RNA, and compartmentalisation of both viral and host factors required for replication [46].

As with cell entry events, additional host factors are increasingly recognised to play a role in viral replication. Recently, the liver-specific microRNA miR-122 has been found to interact with the 5'UTR to enhance HCV replication [55], perhaps by protecting the HCV RNA from degradation by the host cell [56], or by enhancing the interaction between the IRES and ribosome [57]. Furthermore, proteins of the cyclophilin family appear critical for HCV replication. Pharmacological inhibition of cyclophilin A potently inhibits HCV replication *in vitro* [58]. The exact role of cyclophilin proteins in HCV replication is not yet clear, although an interaction with NS5A has been postulated [59, 60], and more recent

data suggests that interaction with cyclophilin A increases NS5A-RNA binding capacity, which is reduced by cyclophilin inhibitors [61]. Interestingly, the role of NS5A in formation of the membranous web also appears to require cyclophilin activity [53, 62]. Finally, another more recently identified cellular factor necessary for HCV replication is phosphatidylinositol 4-kinase type III- α (PI4KIII- α). Again, this enzyme appears to interact with the viral NS5A and may be required for structural integrity of the replication complex [63, 64].

Conversely, viral proteins also interfere with host cell signalling, in particular activation of the innate immune system. Binding of viral dsRNA with toll-like receptor 3 (TLR3) or the cytosolic RNA helicase retinoic acid inducible gene-1 (RIG-I) triggers induction of type-1 interferons through separate signalling pathways. The NS3/4A complex can interfere with induction of type-1 interferons in response to dsRNA sensing through both pathways, by degradation of the TLR3 adaptor protein TRIF and the RIG-1 adaptor protein Cardif [65, 66]. This interference likely contributes to viral immune evasion, and both the establishment and maintenance of chronic viral infection.

The events governing virus particle assembly and release are incompletely characterised. Assembly appears to be a complex, multistep process involving a number of cellular factors as well as most of the viral components. Following its cleavage from E1 by signal peptide peptidase, the core protein moves from the endoplasmic reticulum membrane to associate with cytosolic lipid droplets [67]. Assembly may be initiated on these lipid droplets, or on the ER membrane in close proximity with core-associated lipid droplets [68]. NS5A appears critical to

viral assembly, and this role is mediated by domain III of the non-structural protein [69]. Recruitment of NS5A to low density membrane fractions around cytosolic lipid droplets is promoted by CK1-dependent NS5A hyperphosphorylation, facilitating interaction with core and nucleocapsid assembly [70]. The maturation and release of viral particles is tightly linked to the VLDL pathway. Infectious virions produced *in vivo* or in cell culture differ in their buoyant density and lipid association profiles, supporting the notion that exploitation of cellular lipid pathways by HCV for assembly and release results in the production of lipo-viral particles [68]. The precise mechanism by which this occurs, however, remains to be described.

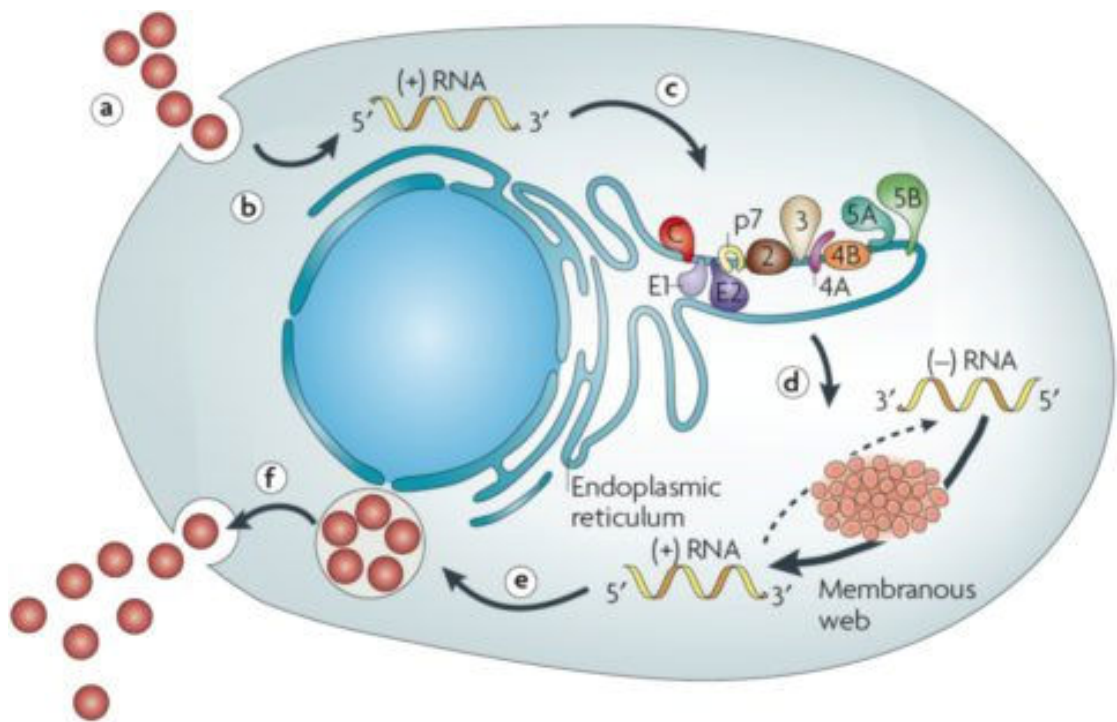


Figure 1-3. Schematic representation of current understanding of the HCV replication cycle. Entry in to the cell (a) is followed by cytoplasmic release and uncoating (b). Initiation of translation and polyprotein processing (c) and RNA replication (d) precede virus packaging and assembly (e) and finally release (f). From Moradpour *et al* (2007) [46].

1.3 HCV quasispecies diversity

Importantly, the viral NS5B RNA-dependent RNA polymerase (RdRp) has no proof reading capacity. Combined with the high replication rate of HCV (an estimated 10^{12} virions are produced in an infected individual per day [71], this results in a high rate of viral variation. Consequently, within an infected individual HCV exists as a closely related viral quasispecies, usually centred round a dominant sequence. The genetic variation of the quasispecies swarm may be up to 10% [72]. Sequence variation is distributed equally throughout the genome, with the exception of the 5'UTR and core regions which are relatively well conserved, and a region within the E2, the highly variable region (HVR), where greater than average genetic diversity is observed [72]. The viability of many of these mutated viruses is not clear. The need to establish a consensus sequence, which led to the successful development of the two existing HCV replication models (the replicon system and JFH-1 cell culture system), suggests that many individual sequence variants may be non-viable [28, 73].

It is widely hypothesised that the evolution of viral quasispecies is driven by continued selective pressure from the host immune system, in a constant process of immune escape. A dynamic viral quasispecies appears important in both establishment of chronicity in acute HCV infection [74, 75] and also in continued escape from antibody and T cell responses in chronic HCV infection [76, 77].

The existence of viral quasispecies, lack of proof reading capacity of the HCV RdRp and rapid viral turnover also becomes important in the context of new

antiviral therapies directly targeting viral non-structural proteins (direct-acting antivirals, DAAs). It is possible for isolates with pre-existing mutations conferring resistance to DAAs to be present within the viral quasispecies population of an infected individual, although to-date the clinical significance of these pre-existing resistance-conferring variants (RAVs) is uncertain (see for example [78-81]. This effectively precludes monotherapy with DAAs, due to the high risk of emergence and selection of RAVs during single agent therapy, leading to treatment failure [82, 83] (discussed in more detail below).

1.4 Tools to study HCV replication

For many years, study of the HCV lifecycle has been hampered by its poor growth in culture and lack of a small animal model of infection. Early *in vitro* models involved generation of virus-like particles expressing HCV envelope proteins in insect cells [84]. These were valuable for early studies of cell entry and investigation of host immune responses to HCV infection [12, 85-87] however they were not replication competent and so could not be used to study other aspects of the life cycle. Furthermore, generation in non-eukaryotic cells raised concerns regarding relevance of glycoprotein expression to HCV infection of humans. Significant steps forward came with generation of HCV pseudoparticles (HCVpp) in mammalian cells, and then with the development of the HCV replicon and the discovery of the JFH-1 strain, a unique HCV strain which replicates and produces infectious virions in tissue culture.

1.4.1 HCV pseudoparticles

HCV pseudoparticles (HCVpp) comprise HCV envelope proteins expressed on a lentiviral core [88]. They differ from the early virus-like particles in that they are generated within mammalian cells, 298T cells, and so may more closely replicate HCV virions generated in human infection. HCVpp have been enormously useful in identification of HCV entry factors, as well as some seminal studies describing host antibody responses to HCV infection [75, 89]. However, as they do not contain HCV replicative machinery, they cannot be used to investigate viral life cycle beyond cell entry.

1.4.2 HCV replicon

The HCV replicon system is based on autonomous replication of subgenomic particles derived from a genotype 1b strain. The replicons comprise the HCV 5'UTR, a neomycin selection marker, a heterologous IRES allowing efficient expression of viral proteins NS3 to NS5B, and the HCV 3'UTR. Transfection of these replicon RNA molecules into Huh 7 cells permits selection of a cell line expressing high levels of HCV RNA and viral proteins, although infectious virions are not produced [73]. Replication in this system is critically dependent on host cell permissiveness and acquisition of cell culture adaptive mutations [90-92]. The latter in particular may limit applicability to HCV infection *in vivo*. Attempts to create replicons of viral genotypes other than 1 and 2 proved technically challenging, requiring considerable tissue adaptation or incorporation of elements of the G1b structure [93-95]. More recently, G3a and G4a replicons have been described [96-98], extending the range of genotypes which can be studied.

1.4.3 Cell culture produced HCV

To-date, only one HCV strain has been identified which will replicate to significant levels *in vitro*. Japanese fulminant hepatitis-1 (JFH-1) is a G2b HCV strain which replicates in tissue culture, without the need for adaptive mutations, producing virions which are infectious to Huh 7 cells (cell culture produced HCV, or HCVcc) [28, 99]. This unique strain was derived from virus taken from a Japanese patient with fulminant hepatitis, a highly atypical clinical presentation of acute HCV infection. Recombinant strains based on the JFH-1 G2b structure but expressing NS3 and NS4A proteins from other viral genotypes have been engineered. However, it has not proved possible to generate infectious recombinant viruses expressing NS3/4A from all genotypes/subtypes, and many of these recombinants require acquisition of further adaptive mutations in order to replicate robustly in culture [100, 101].

1.4.4 Limitations of current replication models

The replicon, HCVpp and HCVcc systems have allowed huge advances in understanding of the HCV lifecycle and anti-HCV drug development. However, their applicability to HCV in infected individuals has some critical limitations. Replication is limited to the human hepatoma cell line Huh7 and its subclones which differ from primary human hepatocytes (PHH) in a number of ways, including poor differentiation, a lack of polarisation and aberrant lipoprotein production compared to PHH [102]. Development of G3a and G4a replicons extended the range of viral genotypes that could be studied [97, 98, 103], and chimeric variants have been extensively used in the analysis of resistance to novel antiviral agents. However, these systems are of limited value in the analysis

of replication and drug sensitivity of an individual patient's virus. For example, to define the drug sensitivity of a single viral isolate requires subcloning of the gene for each direct acting antiviral agents' (DAA) target and insertion into a subgenomic replicon [104]. Since it is difficult to generate replicons with more than one modified locus, studies of multi-drug combinations are difficult.

A number of alternate model systems have been investigated to better replicate primary infection but these do not have widespread applicability, mainly due to costs, technical difficulty or limited availability of materials. Immunodeficient mice engrafted with human hepatocytes can support HCV infection but are expensive and technically challenging to maintain [105]. Human induced pluripotent stem cells (hiPSC) and human embryonic stem cells (hESC) can be induced to differentiate into hepatic-like cells (HLCs), which support replication of the HCV replicon, HCVcc and a limited number of primary patient-derived strains. As the nomenclature suggests, HLCs exhibit some differences to mature primary human hepatocytes and these have been compounded by differences in source cell type and differentiation protocol used (reviewed in [106]). Direct infection of PHH by HCV-infected sera has recently been described, although only a minority of sera replicated sufficiently in this model for use in experimental protocols and the technique is difficult to reproduce due to limited availability of cells [107]. A reliable, high throughput model to study replication and drug sensitivity of patient-derived HCV remains lacking.

1.5 Extrahepatic HCV replication

Whilst the liver is the primary site of HCV replication, HCV RNA has been detected in non-hepatic cell types including CNS, bone marrow and peripheral blood mononuclear cells (PBMCs) [108]. The presence of HCV genomes alone does not confirm viral replication and determining whether HCV can replicate in extrahepatic sites has proved challenging. Demonstration of HCV replication has relied mainly on detection of HCV negative strand RNA by PCR or in situ hybridisation (ISH) or visualisation of HCV non-structural proteins by immunofluorescence or immunohistochemistry. As the RNA negative strand is a replicative intermediate and the non-structural proteins are not present in the mature virion, presence of either, or ideally both, is indicative of viral replication in the cell. The challenges faced by investigators using these techniques have been several fold. Firstly, HCV RNA is present within cells at very low levels and negative strand RNA degrades rapidly after tissue sampling [109]. When detected, negative strand HCV RNA is present at levels 10 – 1000-fold lower than positive strand RNA, challenging the detection capability of even the most sensitive PCR assays [108]. The technique of amplifying the RNA negative strand has itself been widely criticised, in particular due to the risk of false positive results due to self-priming by the RNA or priming by fragments of RNA or DNA present in the reaction [110]. In response to these concerns recombinant Tth polymerase (derived from *Thermus thermophilus*) was adopted in more recent studies as its catalytic activity remains intact at higher temperatures than standard Taq polymerase, reducing the rate of false priming events [111]. ISH is less sensitive for RNA detection than PCR, and its sensitivity can be further reduced if the number of single stranded RNA molecules is reduced by binding of positive and

negative strands [108]. Detection of NS proteins may also be problematic, as they are present in low concentration within cells. An antibody of high sensitivity is required to avoid false negative results.

Given these challenges and limitations, the question of whether HCV can replicate in extra-hepatic sites has remained controversial. However, a considerable number of investigators using a range of these techniques have demonstrated negative strand RNA and NS proteins in extra-hepatic sites. Added to this, compartmentalisation of HCV variants has been described, suggesting adaptive sequence mutations to facilitate persistence in non-hepatic cells (see below). Taken together, this evidence is suggestive of low-level extra-hepatic HCV replication.

1.5.1 Detection of HCV in PBMCs

The presence of replicating HCV in PBMCs taken from patients with chronic HCV infection (demonstrated by negative strand RNA, viral protein detection or both) has been reported by several investigators [112-116]. HCV replication has also been described in PBMCs of patients who have cleared the virus from serum following treatment [116-120] although others have failed to confirm this finding [115]. Whilst HCV replicative forms have been demonstrated in B and T lymphocytes, monocytes/macrophages and dendritic cells, HCV appears to be detected most frequently in B cells and monocytes/macrophages [116, 119]. Replicative forms of the virus were generally detected in cells from some but not all participants. Whether this reflects insufficient assay sensitivity to detect very

low level viral replication, or patient- or virus-specific factors governing entry and replication in these cells, is unknown.

1.5.2 Infection of PBMCs *in vitro*

As the primary site of HCV replication is the liver, studies on cell entry have focused on receptors and co-factors mediating entry into hepatocytes. These receptors may be present on other cell types; for example, neuroendothelial cell lines have recently been reported to express CD81, SR-B1, claudin and occludin, and to be permissive to HCV replication *in vitro*. These receptors have also been demonstrated on neuroendothelial cells *in vivo*, and may represent the means by which HCV gains entry into the CNS [121]. Alternatively, HCV may utilise alternate receptors to gain entry into non-hepatic cells.

Monocytes/macrophages and B cells express CD81 and SR-B1. As these cells do not form tight junctions, they do not express claudin-1 or occludin [122]. Given its function in binding of antibody-antigen complexes, the Fc receptor (FcR) is an attractive candidate receptor for HCV entry into PBMCs. Upregulation of FcR expression by monocytic cell lines enhances HCV uptake which can be abrogated by FcR blocking, implicating the FcR as a route of HCV entry into these cells, although the effect of cytokine stimulation on other HCV entry receptors was not described [123]. Furthermore, CD5 has recently been implicated in HCV cell entry into primary and immortalised T cells [124].

Primary or immortalised B cells, T cells and monocytes/macrophages can be infected with patient-derived HCV *in vitro*, and appear to permit low-level

replication, as evidenced by detection of HCV negative strands, demonstration of nonstructural proteins within the PBMCs by immunofluorescence, or an increase in HCV RNA during the period of incubation [125-129]. Thus far, efforts to infect B lymphocytes, T cell or monocytes/macrophages *in vitro* with HCV produced in cell culture (HCVcc), which is infectious to hepatoma cell lines, have proved unsuccessful [122, 124, 130]. This highlights the critical differences between patient-derived HCV and HCVcc which may mean that cell culture-produced strains cannot be used for studies of extrahepatic cell entry and replication, and will make investigation of this area of HCV biology more challenging. It also supports the concept that HCV may enter non-hepatic cells using alternate mechanisms to those used for hepatocyte entry. It is interesting to note that the HCVcc strain JFH-1 is derived from a viral isolate which caused a fulminant hepatitis, an extremely unusual presentation of acute HCV infection [131]. In this context, it is tempting to speculate that infection of monocytes, or other PBMCs, may mitigate the clinical impact of acute HCV infection.

1.5.3 Compartmentalisation of HCV variants

Compartmentalisation of HCV, whereby genetically distinct viral variants are found in non-hepatic cells and in plasma, has been cited as evidence for viral replication in these cells [132, 133]. The quasispecies detected in PBMCs appear distinct from those associated with immunoglobulin, making it less likely that the variation simply reflects differences in immunoglobulin-bound virus endocytosed into cells [132]. The possibility that compartmentalisation may be due to selective adsorption of a minor variant in the quasispecies onto the surface of cells rather

than infection of the cells, has been effectively excluded through adsorption studies [134].

Compartmentalisation occurs systematically within the E2 highly variable region, and these variants are stable over time [132, 133]. However, as this region is implicated in interaction with entry receptors, it remains possible that distinct sequences reflect a restriction on cell entry rather than replication and acquisition of adaptive mutations. The discovery of distinct mutations within the 5'UTR internal ribosomal entry site (IRES) of virus strains isolated from non-hepatic cells is more suggestive of adaption to this environment.

Given its critical role in ribosomal binding to initiate translation of the viral genome, the IRES, located in the 5'UTR, is one of the least variant regions of the HCV genome. However a quasispecies distribution of 5'UTR variants within the plasma of patients with chronic HCV has been described [135]. These variations are likely to be of biological significance as small variations in the nucleotide sequence in this region can produce considerable differences in translational efficiency. Interestingly, a given sequence alteration could enhance or reduce translational efficiency in different cell lines *in vitro*, suggesting that interactions between the IRES and specific cellular factors may be important in governing translational efficiency, and thus cell tropism [135-137].

Long term culture of HCV in two different lymphoblastoid cell lines resulted in selection of three nucleotide substitutions within the 5'UTR (G(107)→A, C(204)→A and G(243) →A) [129]. The fact that the same substitutions were

selected during growth in two distinct cell lines suggests that they conferred favourable characteristics for replication in these lymphoid cells. Indeed, these substitutions enhanced translational efficiency in some, but not all, of a panel of lymphoid cell lines, compared to that seen in human hepatoma cells [137]. The same substitutions have been identified in viral sequences obtained from PBMCs and monocyte-derived dendritic cells from patients with chronic HCV [136, 138], and the C(204)→A , G(243) →A variant has been identified in post-mortem brain samples from patients with chronic HCV. In this case, the translational efficiency of these IRES variants was found to be lower in cultured microglial cells than human hepatoma cells. One interpretation of this finding is that selection of this variant may contribute to viral latency by permitting low-level viral replication, insufficient to trigger a host immune response [139]. Clearly, translational efficiency measured *in vitro* using bicistronic dual luciferase reporter systems expressing the IRES sequence of interest in immortalised cell lines may exhibit critical differences to factors governing translational efficiency *in vivo*. This makes it difficult to draw conclusions regarding the functional significance of these IRES substitutions. The selection of these variants does however provide further evidence suggesting that low-level replication of HCV can occur in non-hepatic cells.

1.5.4 Clinical significance of extrahepatic replication

The presence of viral genomes in extrahepatic sites has been speculated to lead to a number of clinical features seen in chronic HCV infection. For example, the presence of HCV RNA in the CNS is postulated to be linked to disproportionate fatigue and neurocognitive symptoms reported by patients [140, 141]. HCV

infection is associated with a number of extra-hepatic clinical syndromes, including mixed cryoglobulinaemia, non-Hodgkin lymphoma, lichen planus and Sjogren's syndrome. The pathogenesis of many of these conditions in the context of HCV infection is thought to be multifactorial, related to accumulation of circulating immune complexes and also to stimulation of monoclonal rheumatoid factors by the virus. Given the predominantly autoimmune nature of these conditions it is widely postulated, although controversial, that HCV lymphotropism may also contribute [142, 143].

Replication-competent extrahepatic HCV may additionally represent a reservoir for propagation of infection of hepatocytes, reinfection of the grafted liver after transplant or possibly even relapse after antiviral therapy, if the extrahepatic virus occupies a site which is relatively protected from the effects of treatment. *In vitro*, HCV associated with B lymphocytes could be successfully transferred to hepatoma cells, and infection of the hepatoma cells was enhanced by this route compared to uptake of free virions [130]. This may suggest a mechanism for propagation of infection, for re-infection of the grafted liver after liver transplantation, or for relapse after antiviral treatment. Where liver transplant recipients are chronically infected with HCV at the time of transplantation, the primary source of re-infection of the grafted liver appears to be serum although PBMC strains have also been implicated [144]. One study which examined such patients found identical sequences in the serum post-relapse as in the explanted liver, however the number of patients included was extremely small, and the cell types harbouring virus within the liver, whilst presumed to be hepatocytes, were not actually characterised [145].

Persistence of HCV in PBMCs has been identified as a risk factor for virological relapse after treatment in patients who are co-infected with HIV [146]. However, HIV co-infection appears to enhance HCV replication in PBMCs [147], and so whether HCV persistence in PBMCs during treatment predicts relapse in HCV mono-infected patients remains to be determined.

1.6 Treatment of chronic HCV infection

Until very recently, weekly subcutaneous injections of pegylated interferon- α combined with daily oral ribavirin (pegIFN/RBV) was the established treatment for chronic HCV infection of all viral genotypes following the pivotal registration trials over a decade ago [148-150]. In these trials, a sustained virological response (SVR, defined as undetectable HCV RNA in serum 24 weeks after the end of treatment) was achieved in up to 46% of patients with G1 infection following 48 weeks of therapy. SVR was seen in up to 82% of patients with G2 or 3 infection treated with 24 weeks of therapy, although SVR rates have been somewhat lower in “real world” studies, particularly in patients with G3 infection [151]. Patients with G4 infection have participated in these trials in relatively low numbers, but response rates to treatment are thought to lie somewhere between that of G1 and G2 or 3 infection, and so patients with G4 HCV were generally treated with 48 weeks of therapy. Recent data suggest that treatment responses of G5 and 6 also lie somewhere between that of G1 and 2/3 [152-154].

The development and licensing of the first direct-acting antiviral agents (DAAs) has radically altered HCV therapy over the last few years, and change is ongoing

as further agents are licensed for use. At the time of writing, DAAs targeting the HCV NS3 serine protease (protease inhibitors; PI) are in widespread use for treatment of G1 infection, although are not licensed for treatment of other viral genotypes. Due to an unfavourable side effect profile these first generation PIs have recently been eclipsed in treatment guidelines by therapies based on drug combinations containing the HCV NS5B inhibitor sofosbuvir [155]. However pegIFN/RBV remain, for the time being, important components of HCV treatment regimens, and are the only option for treatment in some parts of the world where DAAs are not readily available, or for viral genotypes where there is currently insufficient evidence to support the use of DAAs.

1.6.1 Interferon- α

The first report of the potential efficacy of interferon- α monotherapy in treatment of chronic HCV infection was published in the late 1980s [156], and it was licensed for treatment of HCV in 1991. Interferon- α is a key cytokine component of the anti-viral innate immune response. It acts through its cell surface receptor to trigger a signalling cascade which culminates in upregulation of transcription of numerous interferon-stimulable genes, and establishment of an anti-viral state within the cell. Anti-viral functions of some of the better characterised interferon-stimulable genes include: indirect inhibition of translation initiation by the protein kinase PKR; RNA degradation by the OAS synthetase family and RNase L; inhibition of RNA synthesis by the family of Mx GTPases; and editing of double stranded RNA by deamination of adenosine to inosine by ADAR1 (reviewed in [157]). Interferon- α also plays a role in activation and direction of the adaptive immune system to assist in the anti-viral response.

Treatment with recombinant human interferon- α as monotherapy for chronic HCV leads to SVR in less than 20% of patients [158]. Addition of polyethylene glycol to the interferon- α molecule to create pegylated interferon- α (pegIFN) has increased the antiviral efficacy, as well as allowing less frequent dosing due to sustained absorption and a longer half-life.

1.6.2 Ribavirin

Ribavirin is a synthetic guanosine analogue, whose exact antiviral mechanism of action is still not well understood. Given alone, it has little effect on serum HCV RNA. In combination with either unmodified or pegylated interferon, however, it substantially enhances treatment response and also appears to reduce the risk of virological relapse after treatment [148, 158]. Its antiviral effect likely occurs through a number of mechanisms. These may include inhibition of RNA synthesis, and thus viral replication, by inhibition of the cellular enzyme inosine monophosphate dehydrogenase; direct inhibition of the viral RNA-dependent RNA polymerase; promotion of lethal mutagenesis in the HCV RNA genome; immunomodulation and promotion of a Th-1 cytokine response pattern; and potentiation of the antiviral action of interferon- α (reviewed in [159]). In treatment of genotype 1 HCV infection, higher weight-based ribavirin dosing produced better outcomes than fixed dosing (1.0 – 1.2 g/day or 0.8 – 1.4 g/day when used in combination with pegylated IFN- α 2a or pegylated IFN- α 2b, respectively) [149]. No advantage was seen using weight-based dosing compared to fixed dosing for patients with genotype 2 and 3 infection [149], so these patients are usually

treated with fixed dose ribavirin 0.8 g/day to limit potential adverse events related to ribavirin exposure.

1.6.3 Side effects of pegylated interferon/ribavirin therapy

A major limitation to pegIFN/RBV treatment is the side effect profile associated with these drugs. Common side effects include 'flu-like symptoms (fatigue, headache, pyrexia, myalgia, arthralgia), neuropsychiatric symptoms (insomnia, anxiety, irritability, depression), gastrointestinal symptoms (nausea, anorexia, weight loss), as well as dermatitis, alopecia and dyspnoea [148, 150]. These adverse events were classified as severe in up to a third of patients [149] and a similar number required pegIFN and/or RBV dose modification due to side effects [149, 150]. Up to 16% withdrew from therapy due to adverse events [149, 150]. Since publication of the registration trials, other unusual or severe side effects which have been described include seizures, severe bacterial infections, autoimmune reactions, interstitial lung disease, neuroretinitis, bone marrow aplasia and idiopathic thrombocytopaenia [160]. Laboratory abnormalities associated with pegIFN treatment include neutropaenia and thrombocytopaenia, whilst RBV is particularly associated with haemolytic anaemia. Transaminase flares and thyroid dysfunction may also occur. These may also require dose reduction of pegIFN and/or RBV. Reductions in pegIFN/RBV below 80% of the recommended dose over the duration of treatment have been associated with lower SVR rates [161].

1.6.4 Treatment response

The rate of decline of serum HCV RNA during pegIFN/RBV treatment is characterised by two phases: a rapid early decline occurring within the first 24 - 48 hours of treatment which is thought to reflect clearance of HCV from the blood, followed by a slower second phase of decline, thought to reflect clearance of infected cells. The rate of response to antiviral treatment is predictive of achievement of SVR and the presence or absence of HCV RNA at weeks 4, 12 and 24 of treatment is used to categorise patients according to virological response (Figure 1-4). A slow response to therapy is strongly predictive of a poor likelihood of SVR, and forms the basis of “stopping rules”, where antiviral treatment which is likely to be futile can be stopped early, thereby avoiding unnecessary treatment-related adverse effects and expense.

Patients who fail to respond to pegIFN/RBV therapy most often show either a null response, where serum HCV RNA does not decline to any significant extent in response to treatment, or a relapse, where serum HCV RNA is undetectable at the end of treatment, but becomes detectable again during the follow up period. Patterns of treatment failure show genotypic variation, with null response seen more commonly in patients with G1 HCV whilst relapse is more frequent in patients with G3 HCV. Less commonly, patients may show a partial response to treatment or virological breakthrough during the treatment period. The lower limit of detection of HCV RNA varies between commercially available assays, but is at least <50IU/mL HCV RNA.

Patients who achieve a rapid virological response (RVR, defined as undetectable viral RNA at week 4) have a high likelihood of SVR. Those who do not, however, still have a reasonably good chance of SVR and so lack of RVR is not an indication to stop treatment. However, if the viral RNA has not declined by $\geq 2\log_{10}$ from baseline by week 12 (an early virological response, EVR), treatment should be stopped as the chance of SVR in such patients who complete the course of pegIFN/RBV is less than 2% [148]. Patients with $\geq 2\log_{10}$ decline in viral RNA at week 12 but who still have detectable serum HCV RNA at week 24 should also stop treatment, as the likelihood of SVR in these patients is also very low (less than 3%) [150]. These recommendations are based primarily on studies of patients with G1 HCV, where rates of null response are higher and allow analysis of factors which predict treatment outcome. Null response is less common in other viral genotypes (especially G2 and G3), so whilst the same “stopping rules” are used in treatment of all viral genotypes, little supporting data in non-G1 infection is available.

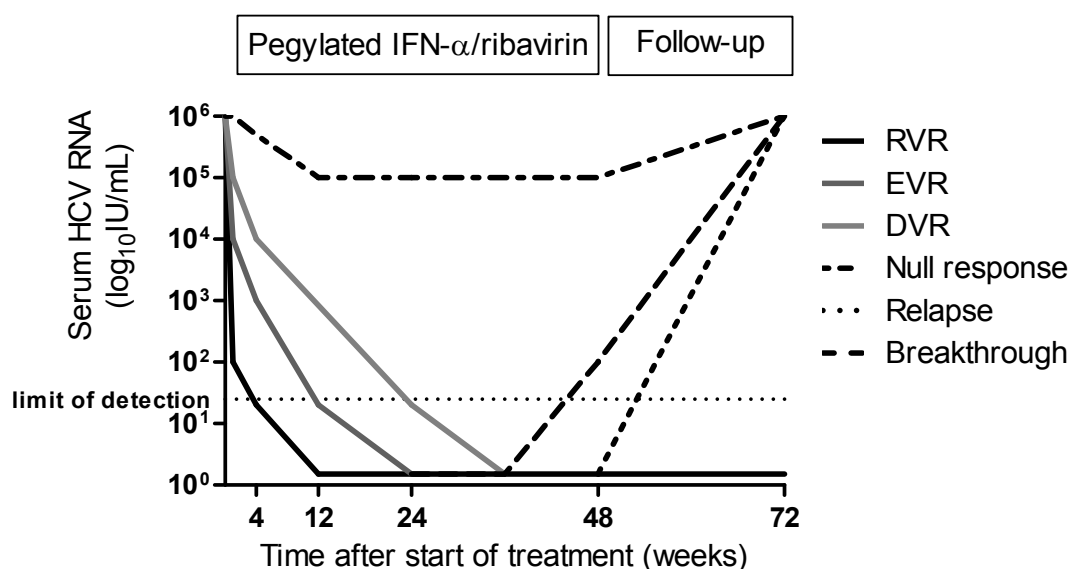


Figure 1-4. HCV response to pegylated interferon/ribavirin treatment. Figure shows different virological responses which may occur during 48 weeks of treatment with pegIFN/RBV, and 24 weeks of follow up after treatment. RVR, rapid virological response; EVR, early virological response; DVR, delayed virological response.

1.7 Predictors of response to pegIFN/RBV treatment

It would be enormously useful to be able to predict which patients will respond to pegIFN/RBV therapy. No single marker can reliably predict the outcome of treatment in an individual, but a number of factors have been identified which indicate “good risk” or “poor risk” for achievement of SVR following pegIFN/RBV therapy. Those most widely used are readily available clinical features, although other host characteristics have been identified which may influence response, including IL28B genotype and baseline expression of interferon-stimulable genes (ISGs). With the exception of viral genotype, virological characteristics which may predict treatment response to pegIFN/RBV have remained elusive. An exception to this is the identification of a region in the NS5A sequence of G1b strains, termed the interferon sensitivity determining region (ISDR). Accumulation of mutations in this region predicts better response to interferon-based therapy in patients with genotype 1b infection [162, 163]. This phenomenon has not been described in patients infected with other viral genotypes.

1.7.1 Clinical features

The strongest clinical predictors of treatment success are HCV genotype and stage of liver fibrosis. To a lesser degree, factors which negatively influence the likelihood of achieving SVR include high baseline viral load (>800,000IU/mL), older age, higher body mass index, insulin resistance, male gender and co-infection with another hepatotropic virus or HIV [160].

Unfortunately, as these patients will benefit most from viral eradication, presence of advanced fibrosis or cirrhosis can substantially reduce SVR rates. This effect

is seen across viral genotypes; response rates of just 34% have been observed in G1 patients with cirrhosis, and as low as 44% in cirrhotic patients with G3 infection [164].

1.7.2 *IL28B* genotype

Host genotype at single nucleotide polymorphisms (SNPs) on chromosome 19 upstream of the *IL28B* gene, coding for interferon- λ 3, has recently emerged as a strong predictor of treatment response to pegIFN/RBV, particularly in patients with G1 HCV infection [165, 166]. Increased prevalence of the poor risk T allele at the rs12979820 SNP appears to explain much of the poorer treatment responses seen in African-American patients with G1 HCV [165]. However, although *IL28B* genotype may assist in estimating the likelihood of response to therapy, its individual predictive value is low as a substantial number of patients with the poor risk allele will still achieve SVR with pegIFN/RBV (around 40% of those heterozygous, and around 30% of those homozygous for the T allele) [165]. Further studies have indicated that *IL28B* genotype is also strongly predictive of treatment outcome in G4 infection, but less so in G2, 3 or 5 HCV, although its predictive value may be stronger in this context in patients with other poor risk features for treatment success [167-170]. The biological mechanism underlying the association of these SNPs with treatment outcome remains unknown. Given their location upstream of the *IL28B* gene, it seems reasonable to speculate that expression of IFN- λ 3 is implicated. The IFN- λ family share a number of characteristics with IFN- α and interact with a distinct receptor to induce expression of interferon stimable genes [171]. Furthermore, they have shown anti-viral activity against HCV *in vitro* and *in vivo* [172, 173]. Conflicting data have

been presented on the association between SNP genotype and IL28B gene expression in healthy volunteers, and this area awaits further study [174].

1.7.3 Interferon stimutable genes

The interaction between IFN- α/β and the type I IFN receptor culminates in the expression of over 300 interferon stimutable genes (ISGs), many of which have yet to be characterised. The overall effect is to create an antiviral state within the cell, and they include proteins with antiviral, antiproliferative and immunomodulatory functions [175]. Some of the better characterised examples include protein kinase R (PKR), adenosine deaminase-1 (ADAR-1), the 2'-5' oligoadenylate synthetases (2-5 OAS)/RNase L system and the MxA family of GTPases. PKR is a serine-threonine kinase which phosphorylates eukaryotic translation initiation factor-2, thus blocking translation of viral mRNAs [176]. 2-5 OAS catalyses the synthesis of 2'-5' oligoadenylates which in turn activate RNase-L. RNase-L then degrades both cellular and viral RNA [175]. ADAR-1 catalyses the deamination of adenosine on double stranded RNA to inosine, destabilising the secondary structure and leading to the accumulation of mutations within the viral genome [175]. Expression of MxA results in degradation of cellular RNA, general repression of protein synthesis and apoptotic cell death [177].

A high level of expression of these and other ISGs in the liver pre-treatment is associated with a poor response to pegIFN/RBV in patients with chronic HCV [178]. Interferon responses seem maximally stimulated and refractory to further induction in these patients, as no further increase in hepatic ISG expression could

be induced following initiation of interferon therapy [179]. Given this association, the role of ISGs which can be measured in the serum has been evaluated as a pre-treatment predictor of treatment response. Interferon inducible protein-10 (IP-10) is a T-cell specific chemokine, the levels of which are elevated in the liver and serum of patients with chronic HCV. Serum IP-10 levels are higher in treatment non-responders than responders [180], and pre-treatment serum IP-10 can be used as a predictive factor of pegIFN/RBV treatment response in patients with chronic G1 HCV infection [181]. Whether there may be a role for pretreatment serum IP-10 as a predictor of treatment response in other viral genotypes is not clear.

1.8 Novel antiviral agents

Greater understanding of the HCV lifecycle and identification of host and viral proteins essential for viral replication has hugely accelerated anti-HCV drug development in recent years. These novel agents either target viral proteins essential for viral replication (direct-acting antiviral agents, DAAs) or host proteins which play a critical role in the viral life cycle.

1.8.1 Direct-acting antiviral agents (DAAs)

The major classes of DAAs either recently licensed or in advanced stages of clinical development are inhibitors of the HCV NS3/4A serine protease, nucleoside or non-nucleoside inhibitors of NS5B, and NS5A inhibitors. Key considerations in use of these drugs are their potency, resistance profile, genotypic coverage and side effect profile.

1.8.1.1 Protease inhibitors (PI)

The HCV NS3 protein has helicase/NTPase activity, and also associates with its obligatory co-factor NS4A to form a serine protease, the NS3/4A. This protease is required for self-cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions during viral replication, but may also inhibit activation of interferon signalling pathways in infected cells [182]. Antivirals targeting this protease may enhance interferon responsiveness as well as inhibiting viral replication, and so it has been an attractive early target for DAA development.

The first two pharmacological inhibitors of the NS3/4A protease licensed for treatment of G1 chronic hepatitis C infection were telaprevir and boceprevir. These first generation protease inhibitors (PI) are orally bioavailable, linear α -ketoamide derivatives which bind covalently but reversibly to the protease active site serine (Ser-139). In clinical trials, these agents demonstrated potent antiviral activity and substantially enhanced SVR rates in patients with G1 HCV when given together with pegIFN/RBV [183-186].

A significant limitation of these first generation PIs is their side effect profile, most notably significant rash (telaprevir) and anaemia (boceprevir). Twice or thrice-daily dosing means a significant pill burden for patients, and the requirement to take these medications with food (and consume a high fat diet) is an additional burden for patients struggling with side effects which often include nausea and loss of appetite.

A further limitation to the use of these PIs is their relatively low genetic barrier to resistance, meaning that a single amino acid substitution within the target site is sufficient to confer significant drug resistance, and this precludes PI monotherapy. For example, a number of mutations conferring varying degrees of telaprevir resistance have been identified *in vivo* and characterised by *in vitro* assessment, particularly at positions 36, 54, 155 and 156 of the NS3 protease catalytic domain [187]. Virological breakthrough or plateau in patients treated with telaprevir monotherapy for fourteen days was associated with emergence of viral strains harbouring mutations at these positions [83, 187]. Amino acid mutations T54A, V36A/M, R155K/T and A156S were associated with low-level resistance to telaprevir in the replicon model, whilst A156T/V and double mutations V36M+R155K and V36M+A156T conferred high-level resistance [187]. Mutations V36M and R155K/T have only been detected in G1a isolates, presumably because only one nucleotide change is required to generate these amino acid substitutions in G1a viral genomes. Differences in the nucleotide composition between subtypes 1a and 1b mean that two nucleotide changes are required to generate the same amino acid substitutions in G1b strains, providing a higher genetic barrier to the development of these particular RAV. Of note, cross-resistance between NS3/4A protease inhibitors has been described, and persistence of selected protease-resistant variants could preclude future treatment with other agents in this class. In particular, mutations at position 155 appears to confer resistance across most PIs in development [188].

The second wave of PIs, now licensed or in advanced clinical development, aimed to achieve comparable or superior potency but with improved genotypic

coverage, a more favourable dosing schedule and side effect profile and improved resistance profile. Simeprevir is a second generation PI which has recently been licensed for treatment of G1 chronic HCV, and at the time of writing a number of other agents (including danoprevir, faldaprevir, asunaprevir and grazeprevir) are in advanced clinical development. In combination with pegIFN/RBV, these agents enhance SVR rates to levels comparable with telaprevir and boceprevir in patients with G1 HCV, with improved tolerability compared to first generation drugs [80, 81, 189-197]. The pharmacokinetics of simeprevir and faldaprevir allow once-daily dosing. Genotypic coverage may be broader than first generation PIs, but the extent to which these drugs will be useful in patients with non-1 HCV genotypes remains uncertain due to the small numbers treated to-date [196, 198-201]. Importantly, G3 HCV remains untreated by this class. Although grazeprevir shows activity against G3 HCV at high doses, in practice this may be limited by drug toxicity [202]. RAVs at positions 155, 156 or 168 confer cross-resistance to nearly all the second generation PIs except grazeprevir, which may make it a promising new agent for patients who have previously failed PI-containing treatment [203].

1.8.1.2 Polymerase Inhibitors

Inhibitors of the HCV NS5B polymerase can be divided into two categories; nucleoside analogue inhibitors (NI) and non-nucleoside inhibitors (NNI).

1.8.1.2.1 Nucleoside analogue inhibitors

Nucleoside analogue inhibitors (NI) compete with the natural nucleoside triphosphate substrate of the HCV RNA polymerase and are incorporated into the

nascent RNA chain, thereby causing chain termination. In early clinical studies, drugs of this class show less potent antiviral activity than PI or NNI [188]. However, significant advantages are their broad genotypic range and relatively high barrier to development of resistance [188, 204]. These drugs bind tightly to the NS5B active site, which is highly conserved across genotypes. Mutations at the active site which would confer resistance also significantly impair viral fitness and replicative capacity [10, 188]. The leading drug in this class, sofosbuvir, has pan-genotypic antiviral activity [205] and has recently been licensed for treatment of HCV G1, 2, 3 and 4. In combination with pegIFN/RBV, sofosbuvir enhances SVR in patients with G1 or G4 HCV compared to pegIFN/RBV alone [206]. The potency of sofosbuvir permits shortening of treatment for patients with G1 HCV to just 12 weeks with little impact on SVR. Unlike PI-based therapies, no difference was seen in treatment outcome between patients with G1a and G1b HCV [205]. Sofosbuvir is well tolerated with few significant adverse events reported, and is dosed once daily. In contrast to PIs, where treatment failure is predominantly through virological breakthrough or (less commonly) primary non response, treatment failure following sofosbuvir-containing treatment is predominantly through relapse. Resistance associated with the S282T mutation has been identified *in vitro* but reports of this variant in patients treated with sofosbuvir-containing regimens are rare. No other NS5B variants conferring resistance in the replicon model have been identified amongst patients who have failed sofosbuvir-containing therapy in clinical trials [207]. Thus, similar to treatment with pegIFN/RBV, mechanisms underlying relapse following sofosbuvir-containing treatment remain to be defined.

1.8.1.2.2 Non-nucleoside inhibitors

In contrast to NI, non-nucleoside inhibitors (NNI) are a heterogeneous group which inhibit the HCV NS5B by binding to one of at least four different allosteric sites, resulting in conformational change of the protein. Similar to other viral polymerases, the NS5B has a characteristic right-hand conformation with palm, finger and thumb domains. NNIs can be subdivided according to their binding site. Site 1 inhibitors bind to the benzimidazole thumb 1 domain, whilst site 2 inhibitors bind to the thiopene thumb 2 domain. NNI site 3 and 4 inhibitors bind to the benzothiadiazine palm 1 and benzofuran palm 2 sites, respectively. In theory, as they utilise different binding sites, these agents could be combined, or used sequentially to manage the development of resistance [10]. In general, these agents show low to average antiviral potency [188]. Unfortunately, they have a low genetic barrier to resistance, and mutations conferring resistance do not necessarily lead to a poorly replicative virus [10, 188]. Pre-existing variants harbouring mutations which confer resistance *in vitro* to NNIs, but not NIs, have been described in serum of HCV G1 infected patients [208, 209]. Furthermore, these allosteric sites are less well conserved between viral genotypes than the enzymatic active site, leading to a risk of varying antiviral efficacy between genotypes and perhaps even between subtypes [188, 209]. Concerns regarding both the low barrier to resistance of these agents, and their relatively low antiviral potency compared to other classes of DAA were borne out in Phase II study of the NNI filibuvir in combination with pegIFN/RBV which showed high relapse rates and no enhancement of SVR compared to pegIFN/RBV [210]. The focus for potential further development of these agents is likely to be as part of multi-drug interferon-free regimens.

1.8.1.3 NS5A inhibitors

Development of DAAs targeting the HCV NS5A has progressed more slowly than for drugs targeting the NS3/4A protease or NS5B polymerase, largely due to the lack of enzymatic activity of the NS5A protein and consequent difficulties in drug candidate screening. Despite this, a small number of agents are now in use, with daclatasvir, ledipasvir and ombitasvir licensed or in advanced clinical development. In early clinical studies daclatasvir showed potent antiviral activity, but a relatively low genetic barrier to resistance; single amino acid mutations appear sufficient to confer reduced drug sensitivity [211, 212]. Considerable NS5A sequence diversity between HCV genotypes suggests that antiviral efficacy of drugs targeting this region may differ between genotypes, and early data suggested daclatasvir may have greater efficacy against G1b than G1a strains [212, 213]. This has been supported by Phase 2 data where SVR following daclatasvir with pegIFN/RBV was higher amongst patients with G1b HCV than G1a [214]. Furthermore, *in vitro* data have suggested that HCV G2 and 3 may have a lower barrier to development of daclatasvir resistance than other viral genotypes [215]. However, clinical data have demonstrated efficacy in patients with HCV G2/3. The addition of daclatasvir to pegIFN/RBV treatment enabled shortening of therapy for patients with G2/3 HCV to 12 weeks, with non-inferior SVR rates to 24 weeks of pegIFN/RBV alone [216]. Given their high antiviral potency but relatively low barrier to resistance development, NS5A inhibitors are attractive candidates for combination therapy as part of IFN-free treatment regimens.

1.8.2 Host-targeting antiviral agents

A multiplicity of host factors are increasingly recognised to play a role in the viral life cycle, from cell entry and replication to packaging and cell egress. These cellular factors provide additional potential targets for antiviral therapeutics, and could potentially be combined with DAAs in the quest for interferon-free drug regimens. They have the advantage of a higher barrier to development of resistance than viral targets, although the possibility of deleterious effects as a result of inhibiting essential host cell factors must also be considered.

Proteins of the cyclophilin family appear critical for HCV replication. The exact role of cyclophilin proteins in HCV replication is not yet clear, although an interaction with NS5A has been postulated [59, 60]. Pharmacological inhibition of cyclophilin A potently inhibits HCV replication *in vitro* [58]. The cyclophilin inhibitor alisporivir showed pan-genotypic activity, although with higher efficacy in genotypes 2 and 3 than 1 or 4 [217]. Although HCV variants with NS5A mutations conferring alisporivir resistance had been identified *in vitro*, rates of virological breakthrough in clinical trials are low [218]. Development of this drug was delayed following a small number of cases of pancreatitis, including one fatality, in patients receiving alisporivir in combination with interferon. It retains the potential to be a valuable backbone in interferon-free regimens.

Recently, the liver-specific microRNA miR-122 has been identified as a host factor which enhances HCV replication [55]. MicroRNAs are small (18-22 nucleotides) non-coding RNA molecules which regulate mRNA expression. MiR-122 appears to interact with the 5'UTR to enhance HCV replication, perhaps by

protecting the HCV RNA from degradation by the host cell [56], or by enhancing the interaction between the IRES and ribosome [57]. Miravirsen is a locked nucleic acid–modified phosphorothioate antisense oligonucleotide which targets miR-122. As the miR-122 binding site is well-conserved, it would be expected to show a broad genotypic range of anti-viral activity, and this has been corroborated *in vitro* [219]. A recent proof-of-concept study in patients with chronic genotype 1 HCV demonstrated moderate anti-viral activity with no emergence of resistant strains over a 4 week dosing period [220].

As our knowledge of the mechanisms governing the HCV life cycle increases, further potential drug targets may emerge. For example, another recently identified cellular factor necessary for HCV replication is phosphatidylinositol 4-kinase type III- α (PI4KIII- α). This enzyme appears to interact with the viral NS5A and may be required for structural integrity of the replication complex [63, 64]. However, at present it is unclear whether it will be possible to design a sufficiently specific and potent inhibitor of this enzyme whilst avoiding significant host toxicity [44].

An alternative approach is modification of existing HCV treatments (pegIFN and ribavirin) to increase efficacy and, more importantly, tolerability. Interferon lambda has similar intracellular antiviral effects to interferon alpha, but affects fewer cell types due to differences in receptor distribution. Phase 1 studies support the hope that this may translate to equivalent antiviral efficacy with a reduced side effect profile [221]. However, further clinical trials comparing pegylated interferons alpha and lambda have been put on hold given the recent

rapid development of DAAs and likely imminent arrival of all-oral treatment regimens.

Ribavirin is associated with anaemia, often managed by ribavirin dose reduction, which could compromise achievement of SVR. Taribavirin is an oral prodrug of ribavirin which is not concentrated in erythrocytes. A comparison of weight-based taribavirin with ribavirin did show less anaemia, but no improvement in SVR rates amongst those receiving taribavirin [222]. Whether there may be a role for taribavirin in future combination therapy is not yet clear. It may perhaps prove useful in combination with DAAs in treatment of patients at particular risk of anaemia, such as those with renal impairment.

1.8.3 Interferon-free treatment regimens

The ultimate aim of anti-HCV drug development is to achieve a well-tolerated, all-oral treatment regimen which will cure all patients with a short course of therapy. Many of the side effects associated with current HCV treatment are caused by interferon, and so development of interferon-free regimens is a logical first step in improvement of treatment tolerability. Proof-of-concept of interferon-free HCV treatment was provided by the INFORM-1 trial, in which potent viral suppression was seen over 14 days of treatment with the PI danoprevir and NI mericitabine [223]. Following the experience of drug development in HIV, where DAAs targeting different viral proteins have been combined to minimise viral resistance, a number of interferon-free regimens are now in development for chronic HCV.

Early mathematical modelling suggested that combinations of three drugs would be required for treatment of chronic HCV, to avoid acquisition of viral resistance [224]. In practice, successful treatment outcomes for some patient groups have been observed with 2 drugs, perhaps due to the trade-off between resistance and viral fitness. For example, successful treatment of G1b strains may be achieved by combining a PI with a relatively low barrier drug (NNI/NS5A inhibitor), whereas G1a strains may require a PI plus 2 additional drugs, as they are more susceptible to emergence of relatively fit RAVs at the 155 position. Following this theory, combinations of NI (high barrier to resistance) with 2 lower barrier drugs (NNI/NS5A inhibitor) might be suitable for both G1a and G1b, whilst combining an NI with a PI might be suitable for all patients, regardless of genotype.

Trial data to-date appear to support this model. Combining asunaprevir (PI) with daclatasvir (NS5A inhibitor) achieved high rates of SVR in patients with G1b HCV but less so for G1a [225, 226]. Addition of a third DAA (an NNI) overcame this and produced comparable SVR between G1a and G1b [227]. In Phase 3 studies evaluating a PI (ABT-450), NNI (dasabuvir) and NS5A inhibitor (ombitasvir) with or without ribavirin, SVR was lower in the ribavirin-free arm in patients with G1a than G1b HCV, but comparable amongst G1a/G1b patients treated with quadruple therapy [228-230]. These data highlight potential differences in treatment response to PI-based therapy between G1a and G1b. A “one-size-fits-all” treatment approach has advantage of simplicity but risks over-treating patients who may do well with fewer drugs [231].

Alternatively, a NI could be used as the backbone for interferon-free combination therapy, given the broader genotypic coverage of this drug class than PIs, and relatively high barrier to resistance. Sofosbuvir is particularly attractive for this role, given its high antiviral potency. The potency of the NI in interferon-free combination therapy appears to be important, as a combination of mericitabine (NI) and danoprevir (PI), with or without ribavirin, was associated with high rates of treatment failure, especially amongst G1a patients, and acquisition of danoprevir RAVs [232]. In contrast, a combination of sofosbuvir (NI) and simeprevir (PI), with or without ribavirin, produced high rates of SVR with just 12 weeks of treatment, with or without ribavirin, for non-cirrhotic patients with both G1a and G1b HCV [233].

Given its high antiviral potency, broad genotypic coverage and high barrier to resistance development, sofosbuvir has been investigated in interferon-free regimens with ribavirin alone. At the time of writing, these relatively small studies have suggested that for patients with G1 HCV, outcomes are less favourable than with sofosbuvir, pegIFN/RBV triple therapy, particularly amongst treatment-experienced patients and those with risk factors for treatment failure [234, 235]. In contrast, interferon-free sofosbuvir/RBV appears adequate for patients with G2 HCV, although patients with cirrhosis may have better outcomes with longer durations of therapy than the standard 12 weeks [205, 236, 237]. Regarding G3 HCV, treatment outcomes with sofosbuvir/RBV dual therapy appear inferior to sofosbuvir with pegIFN/RBV, although at present little clinical trial data is available for G3 patients treated with this triple therapy combination [205, 234, 238]. Current treatment guidelines recommend sofosbuvir/RBV dual therapy for

G2 HCV but not for G1 HCV unless no other interferon-free option is available [155]. Patients with G3 HCV in whom interferon is contra-indicated may have good treatment outcomes if the duration of sofosbuvir/RBV dual therapy is extended to 24 weeks, but this is not recommended for patients with cirrhosis or previous pegIFN/RBV treatment failure [155, 237]. The BOSTON trial was conducted recently to clarify optimal treatment regimens for these viral genotypes. Sofosbuvir/RBV for 16 or 24 weeks was compared with 12 weeks of sofosbuvir/pegIFN/RBV in patients with G2 and G3 HCV. In patients with G3 HCV, sofosbuvir/pegIFN/RBV for 12 weeks was superior to sofosbuvir/RBV regimens of 16 or 24 weeks, regardless of treatment experience or cirrhosis [239]. In the future, sofosbuvir-based regimens may be superseded for G3 HCV but for now, inclusion of pegIFN in sofosbuvir-based treatment should remain an option.

Ribavirin also contributes to the significant burden of side effects experienced by patients receiving anti-HCV therapy. To avoid pegIFN and RBV exposure, sofosbuvir has been investigated in combination with the NS5A inhibitor daclatasvir and also combined in a single pill with another NS5A inhibitor, ledipasvir. Both combination therapies have been investigated with and without RBV. Sofosbuvir and daclatasvir appears highly effective in treatment of G1, G2 and G3 HCV, although SVR rates were slightly higher amongst patients with G1 than G2 or G3 HCV. The addition of RBV did not appear to enhance SVR, although it is notable that in this small study the G3 patients who relapsed did not receive RBV [240]. Sofosbuvir/ledipasvir with or without RBV appears highly effective in treatment of G1 HCV, including patients who have previously failed

PI-based therapies [241-243]. However, efficacy is lower in other viral genotypes, particularly G3, although addition of ribavirin improves SVR [244].

Patients who relapse after DAA therapy represent a new and growing population of “hard-to-treat” patients for whom there is a shortage of information regarding best management. One option is to retreat with extended duration of therapy. To explore the efficacy of this, patients with G1 HCV who had previously failed 8-12 weeks of sofosbuvir/ledipasvir-based therapy were retreated with a prolonged course of 24 weeks sofosbuvir/ledipasvir. Overall, 71% of retreated patients achieved SVR12. Retreatment success was influenced by duration of previous therapy (80% SVR12 in patients previously treated for 8 weeks versus 46% in those previously treated for 12 weeks) and the presence of NS5A RAVs at retreatment baseline (100% SVR12 if no RAVs versus 60% if RAVs present). Furthermore, a small number of patients with NS5A RAVs acquired the sofosbuvir resistance-conferring RAV S282T during retreatment [245]. Thus whilst combinations of sofosbuvir and NS5A inhibitor appear promising as highly efficacious, well tolerated interferon-free therapies, the role of RBV in preventing relapse remains uncertain, and the best option for retreatment of patients who fail these IFN-free regimens remains to be established.

1.9 Predictors of response to novel anti-HCV therapies

As anti-HCV drug development has progressed to include ever more potent combinations of antiviral drugs, fewer patients are failing to respond to therapy. However, failure rates are likely to be higher in “real world” settings than in clinical trials, and even 5-10% treatment failure observed in a trial will represent

significant numbers of patients as these treatments become more widely available. Treatment failure risks acquisition of resistance to novel antiviral therapies and as yet it is unclear how great the clinical significance of this may be. In the era of interferon-free treatments and with increasing numbers of drugs available which vary in potency, pan-genotypic efficacy and barrier to resistance, care will need to be taken to avoid generation of “multi-drug resistant” HCV. The ability to predict which patients will respond to which combinations of drugs would be valuable to minimise toxicity, optimise outcomes and rationalise costs.

As with pegIFN/RBV therapy, no single factor exists which allows prediction of treatment outcome to novel antiviral therapies. Viral genotype/subtype and clinical features, particularly presence of advanced fibrosis/cirrhosis, remain important. Other factors, such as IL28B status, previous treatment history, pre-treatment presence of antiviral resistance-associated mutations, may be more important in treatment with some DAAs and drug combinations than others.

1.9.1 Clinical features

As with pegIFN/RBV therapy, the infecting viral genotype significantly affects response to most DAAs. As discussed above, PIs are licensed only for treatment of G1 HCV as they have little clinical efficacy against other viral genotypes investigated in clinical trials [198, 199]. Greater treatment response to PI-based therapies in patients with G1b than G1a HCV has been consistently observed, as is discussed in greater detail above. DAAs with broad genotypic coverage are still more efficacious in some genotypes than others. Dual therapy with sofosbuvir

and ribavirin is highly efficacious in G2 HCV, but less so in G1 and G3 infection [234, 236].

Emerging data also suggest that there may be HCV strain-specific differences in response to DAAs, which are not readily identified by current genotyping or phenotyping assays. For example, although the overall antiviral efficacy of telaprevir in patients with G3 HCV is low, a minority of patients did show some antiviral response [199]. Furthermore, simeprevir efficacy appears heterogeneous amongst patients with G5a infection [246]. This within-subtype variation, demonstrated in very small series, may be due to naturally occurring amino acid changes within the NS3 protease domain [100]. Whether similar strain-specific variation will occur upon treatment with other DAAs, and the clinical importance of this, remains to be established.

The presence of advanced fibrosis or cirrhosis strongly impacts on outcome of most DAA-containing therapies. Although SVR is improved relative to pegIFN/RBV alone, treatment outcomes remain worse in cirrhotic than non-cirrhotic patients treated with PI (telaprevir, boceprevir or simeprevir) and pegIFN/RBV [80, 183-186]. Outcomes for cirrhotic patients treated with IFN-free regimens are inferior for G1 or G3 patients receiving sofosbuvir/ribavirin dual therapy compared to patients without cirrhosis [235, 236]. Initial studies on treatment of G1 patients with sofosbuvir/simeprevir and sofosbuvir/ledipasvir (both regimens with and without RBV) found equivalent outcomes in cirrhotic and non-cirrhotic patients, regardless of inclusion of RBV, although the number of cirrhotic patients was small [233, 247]. A subsequent larger trial, OPTIMIST-2,

investigated sofosbuvir/simeprevir in G1 HCV patients with cirrhosis. This study found SVR rates slightly lower than previously observed, and suggested that factors associated with poor response (IL28B status, previous treatment experience, presence of simeprevir-resistance conferring Q80K variant) may have a greater influence on treatment outcome amongst cirrhotic patients [248].

1.9.2 Previous response to pegIFN/RBV treatment

Many patients being treated with DAA-containing therapy, especially in the early phase of availability, will have previously failed treatment with pegIFN/RBV. The impact of a patient's interferon response is particularly important when administering therapies combining a single DAA with pegIFN/RBV. Patients who are poorly interferon sensitive will effectively receive DAA/RBV dual therapy, and are at high risk of viral resistance and treatment failure, particularly when the DAA is of a class with a relatively low barrier to resistance. This is demonstrated in clinical trials of PI with pegIFN/RBV, where SVR rates are much lower in patients with previous null response to pegIFN/RBV treatment than prior relapse or partial response [183, 186, 190]. However, a previous null response to pegIFN/RBV is not an absolute predictor of outcome of PI-containing triple therapy, as a greater proportion of such patients went on to achieve SVR than those treated with a further course of pegIFN/RBV alone. In an attempt to predict which of these patients may benefit from further treatment with PI and pegIFN/RBV, a four-week pegIFN/RBV lead-in has been used to establish interferon response before commencing PI. In clinical trials of boceprevir-containing triple therapy, $>1 \log_{10}$ decline in viral RNA during the lead-in phase was associated with higher rates of SVR [183, 185, 249, 250]. Conflicting data have been presented on whether the

use of a lead-in enhances SVR following treatment with telaprevir-based triple therapy, although results may have been influenced by clinician selection of patients receiving lead-in or higher patient drop-out due to pegIFN/RBV-related adverse events [186, 251]. Amongst pegIFN/RBV-experienced patients treated with telaprevir and pegIFN/RBV, $>1 \log_{10}$ decline in HCV RNA during a four week pegIFN/RBV lead-in was associated with higher rates of SVR. However, the authors cautioned that response to lead-in should be combined with detailed clinical history of prior pegIFN/RBV treatment response, as a significant number of prior relapsers and partial responders still achieved SVR despite $\leq 1 \log_{10}$ decline in HCV RNA during lead-in [252].

1.9.3 *IL28B* genotype

The strength of association between *IL28B* genotype and treatment outcome appears to decrease in proportion to the antiviral potency of DAA-based regimens [253]. *IL28B* status retains some predictive value in patients receiving PI and pegIFN/RBV, especially treatment-naïve patients [80, 249, 254]. Patients with poor-risk T allele appear to gain the most benefit from addition of PI to pegIFN/RBV therapy, whilst patients homozygous for the good-risk C allele do well with shorter duration of therapy [255]. The strength of the association is lost in treatment-experienced patients, a population which may be enriched for the poor-risk T allele, where previous response to pegIFN/RBV treatment is a much stronger predictor of treatment outcome than *IL28B* status [249, 256]. The association between *IL28B* genotype and treatment outcome also appears lost following treatment with more potent sofosbuvir-based IFN-containing and IFN-free regimens [206, 240, 247]. The importance of *IL28B* genotype in prediction of

treatment outcome is likely to decline as the use of interferon-free treatments becomes more widespread.

1.9.4 Resistance associated variants

As discussed above (Section 1.2.3), the HCV RNA-dependent RNA polymerase lacks any proof-reading capacity, which, combined with the high rate of HCV replication, results in a very high rate of viral mutation. In theory, all single and double mutations pre-exist before treatment in an infected individual, although in practice mutation rates are not equally distributed over the whole genome, and whether a mutation will persist in the quasispecies is also influenced by other factors such as viral fitness and the replication environment [224, 257]. Mutations which confer reduced susceptibility to DAAs (resistance associated variants, RAV) are detectable pre-treatment in a small proportion of treatment-naïve patients (up to 5%) [208, 258], although their clinical significance is uncertain. Baseline RAV conferring decreased susceptibility to telaprevir or boceprevir do not appear to affect SVR rates amongst patients treated with these PIs and pegIFN/RBV, provided patients had a good pegIFN/RBV response [259-262].

The Q80K variant, which confers reduced susceptibility to simeprevir, appears to be of greater clinical significance. In clinical trials, this variant was present at baseline in 23-41% of patients with G1a HCV (it is rarely detected in G1b) and substantially reduced clinical response to simeprevir and pegIFN/RBV in both treatment-naïve and treatment-experienced patients [80, 81, 189]. Screening for the Q80K variant is recommended for G1a patients prior to treatment with simeprevir and pegIFN/RBV, with consideration of alternate therapy if this variant

is identified. Interestingly, replacement of pegIFN/RBV with sofosbuvir in combination with simeprevir appears to negate the impact of the Q80K variant, as sofosbuvir/simeprevir dual therapy produced high SVR rates amongst non-cirrhotic patients with G1a HCV regardless of Q80K status [233]. This benefit appears to be lost in cirrhotic patients treated with sofosbuvir/simeprevir, where SVR rates were higher amongst G1a patients without Q80K than patient with Q80K [248]. The full impact of Q80K mutations in interferon free regimens remains to be determined.

The implications of acquisition of RAV in patients who have failed DAA therapy are also unclear. As HCV is an RNA virus, unlike HIV or HBV, resistant variants cannot be archived as DNA. Sequencing studies suggest that PI RAV, which have relatively poor replicative fitness, are gradually replaced by wild type virus over weeks to months after cessation of the PI. For example, follow-up viral sequencing after telaprevir-based treatment found resistant variants in 77% of patients without SVR by population sequencing. Kaplan-Meier estimates of loss predicted a median time of 10.6 months for G1a patients and 0.9 months for G1b patients to revert fully to wild-type [79]. RAVs occurring after failure of boceprevir-based triple therapy have also been found to decline over time [261]. Of note, these analyses by population sequencing may not detect persistence of RAVs at very low frequencies within the quasispecies. Studies of retreatment of PI treatment failures with DAA/pegIFN/RBV triple therapy have not been performed, and so the clinical significance of previous PI treatment failure in this context is unknown. However, retreatment with an interferon-free regimen using potent antivirals with no overlapping resistance has been explored and appears

promising. The two small studies investigating interferon-free sofosbuvir/daclatasvir and sofosbuvir/ledipasvir both included patients who had previously failed treatment with PI/pegIFN/RBV, and rates of SVR were high [240, 247]. The PI grazoprevir is unique in that it is not inhibited by NS3 RAVs which confer cross-resistance to other PIs. Treatment of patients who had previously failed PI/pegIFN/RBV with grazoprevir, elbasvir (an NS5A inhibitor) and RBV resulted in high SVR rates, regardless of previous PI virological failure. However, treatment-emergent NS3 and NS5A RAVs were reported in the small number of patients who failed therapy [263].

Pre- and post-treatment RAVs conferring reduced susceptibility to other classes of DAA have been described, but the natural history of these variants and their clinical significance is even less clear at present. Unlike PI RAVs, RAVs conferring resistance to NS5A inhibitors appear to be relatively fit and have been found to persist for at least 12 months after therapy [264]. Acquisition of RAVs conferring decreased susceptibility to sofosbuvir (S282T variant) is very rare, but has been described in a patient treated with sofosbuvir monotherapy, where S282T became undetectable (by deep sequencing) by 12 weeks after treatment [207], and after sofosbuvir/ledipasvir dual therapy, in the context of RAVs conferring reduced NS5A inhibitor sensitivity both at baseline and at the time of relapse [247]. In a clinical trial of prolonged (24 weeks) sofosbuvir/ledipasvir retreatment of patients who had failed 8-12 weeks of sofosbuvir/ledipasvir therapy, presence of NS5A RAVs at retreatment baseline was associated with considerably lower SVR rates (100% SVR12 if no RAVs versus 60% if RAVs present) [245]. However, patients with NS5A inhibitor RAVs at baseline did

achieve SVR, so these alone do not appear predictive of response to sofosbuvir/ledipavir therapy [247]. The implications of these variants in future treatment response is currently unknown.

Overall, previous widely used predictors of treatment response (infecting viral genotype, advanced fibrosis/cirrhosis) remain relevant to DAA-based therapies, although less so to highly potent IFN-free regimens. However the number of participants has so far been small, and some cases of treatment failure have been seen. These numbers are likely to become much more significant as the use of such regimens increases. Given the high rates of treatment success with current interferon-free regimens in patients with traditional “poor risk” factors for SVR (e.g. cirrhosis, previous failure to respond to pegIFN/RBV) new strategies may be necessary to identify individuals at risk of treatment failure, particularly those undergoing “second line” therapy, to maximise outcomes and minimise the risk of generating multi-drug resistant HCV.

1.10 Aims and objectives

The hypothesis of this study was that a novel replication system for patient-derived HCV could be developed, using a fusion technique recently established in the group, and that this replication system could be used to predict patient-specific responses to antiviral therapies.

A novel system to study replication of patient-derived HCV has recently been developed within our group. This system is based around fusion of patient-derived monocytes with cultured hepatocytes and has demonstrated that patient-

derived HCV replicates in the hybrid cells. This project aimed to examine whether this viral replication model can be used to predict outcome of treatment with pegIFN/RBV as well as novel antiviral therapies. Given the rapid progress in development of novel antiviral agents, the ability to screen individual patient sensitivity to novel antivirals could be enormously useful, especially for patients who have already failed therapy. Patient-derived monocytes have a number of limitations for such use, including the low yield of cells, difficulty in storing and difficulty transferring cells between laboratories. In this project, we sought to modify the fusion model such that instead of patient monocytes, HCV from patient serum would be “captured” by cell culture monocytes prior to transfer to hepatocytes by cell fusion. We aimed to validate this “capture-fusion” assay as a clinically useful tool to screen patient drug sensitivity. Furthermore, we aimed to study the mechanisms underlying HCV infection of monocytes and which permit viral replication after fusion in the hybrid cells.

Specifically, the project objectives are:

- To develop a novel assay to study replication of patient-derived HCV, using cell culture monocytes to capture HCV from patient serum, prior to fusion with hepatocytes.
- To assess whether this novel “capture-fusion” model for viral replication can be used to predict sensitivity of patient-derived HCV to interferon, ribavirin and novel direct-acting antiviral drugs.
- To test the hypothesis that presence of viable HCV in monocytes at the end of therapy predicts relapse in patients receiving treatment for chronic HCV infection.

- To investigate whether this model can be used to study the mechanisms underlying HCV infection of monocytes.
- To study the mechanisms which permit viral replication in hybrid cells.

2.0 Materials and methods

2.1 Cell lines

2.1.1 Huh7.5 cells

Huh7.5 is a hepatocellular carcinoma derived cell line which is highly permissive to replication of HCV subgenomic constructs and cell culture adapted strains. These cells (kind gift from Charles Rice, Rockefeller Institute, New York) were maintained at 37°C in 75cm³ flasks in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin). Cell passage was performed every 3-4 days, or when the cells reached 80-90% confluence.

2.1.2 Replicon cells

This is a Huh7 cell line which stably expresses a subgenomic, autonomously replicating HCV construct, and was a kind gift from Ralf Bartenschlager (University of Heidelberg, Germany). The construct comprises the HCV 5'UTR, a neomycin resistance marker, a heterologous internal ribosomal entry site and the HCV NS3-NS5B and 3'UTR. The cells were maintained at 37°C in 75cm³ flasks in DMEM supplemented with 10% FCS and neomycin. Cell passage was performed when the cells reached 80-90% confluence.

2.1.3 Huh7-J20 cells

This is a Huh7 cell line which stably expresses a fusion protein comprising enhanced green fluorescent protein and secreted alkaline phosphatase, linked by the HCV NS4A/NS4B cleavage site. These cells were a kind gift from Arvind Patel (Centre for Virus Research, University of Glasgow). The cells were maintained at 37°C in 75cm³ flasks in DMEM supplemented with 10% FCS and

antibiotics (penicillin and streptomycin). Cell passage was performed when the cells reached 80-90% confluence.

2.1.4 THP-1 cells

THP-1 is a human monocyte cell line, derived from a 1 year old male with acute monocytic leukaemia [265]. This suspension cell line was purchased from ATCC (Teddington, Middlesex, UK) and maintained at 37°C in 75cm³ flasks in RPMI supplemented with 10% FCS and antibiotics (penicillin and streptomycin). Cell passage was performed every 3-4 days.

2.1.5 Passage of adherent cell lines

Medium was removed and the cells washed with sterile PBS. The cells were incubated at 37°C with 5x trypsin diluted in PBS for 4 minutes. The trypsin was poured off and the cells removed from the flask by washing with 10mL DMEM plus 10% FCS. 2mL of the resulting cell suspension was transferred to a fresh 75cm³ flask and made up to 20mL with growth medium.

2.1.6 Passage of suspension cell lines

Cells and medium were transferred to a 50mL Falcon tube and the cells were pelleted (5 minute centrifugation at 1200rpm). The medium was poured off, and the cell pellet resuspended in 5mL RPMI. 1mL of this suspension was transferred to a fresh 75cm³ flask and made up to 20mL with growth medium.

2.2 Primary human monocytes

2.2.1 Collection and separation of total peripheral blood mononuclear cells (PBMCs)

40mL peripheral blood was taken into heparinised tubes from patients attending their end of treatment visit for genotype 3 HCV. All patients gave written informed consent to participate, and the protocol was approved by Moorfields and Whittington Research Ethics Committee (reference 09/H0721/25).

Whole blood was diluted 50:50 with RPMI and layered on Ficoll-Paque (GE Healthcare, Buckinghamshire, UK), before centrifugation at 2500rpm for 20 minutes. The resulting PBMC layer was removed to a fresh 50mL Falcon tube and washed twice, by suspension in 50mL RPMI and centrifugation at 1500rpm for 5 minutes. The total PBMCs were counted and frozen in aliquots of 5×10^6 cells/mL in freezing medium comprising 70% RPMI, 20% FCS, 10% DMSO. The cells were frozen at -80°C and then transferred to liquid nitrogen storage until use.

2.2.2 Separation of CD14 (+) monocytes

When ready for use, vials of PBMCs were thawed to room temperature, then washed by slowly adding 10mL cold RPMI and centrifugation at 1200rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 20mL pre-warmed RPMI plus additives (10% FCS and penicillin/streptomycin). The cells were left for at least 4 hours before further manipulation.

CD14(+) cells were isolated from total PBMCs by magnetic separation (Milteyi Biotec, Surrey, UK). Thawed PBMCs were counted, then pelleted and washed

once in MACS buffer (PBS with 2% FCS and 0.5% EDTA). The pellet was resuspended in 80 μ L MACS buffer per 10⁷ cells. CD14 (+) microbeads were added at 20 μ L per 10⁷ cells and gently mixed, before incubation at 4°C for 15 minutes. The cells and microbeads were then made up to 500 μ L with MACS buffer before being passed through a magnetic cell separation column using LS columns (suitable for up to 10⁸ total cells). The column was washed 3 times with 500 μ L MACS buffer. After all the eluate had passed through, the column was removed from the magnet and transferred to a clean Falcon tube, where the isolated CD14(+) cells were washed through in 1mL MACS buffer. The CD14 (+) cells obtained were counted and cell fusion using these cells proceeded immediately. During optimisation experiments (performed by Dr Alia Javaid), flow cytometry found that cell population isolated using this technique comprised 98% CD14+ cells.

2.3 Fusion of primary monocytes or THP-1 cells with Huh7.5 cells

2.3.1 Pre-stimulation of THP-1 cells

Where THP-1 cells were pre-stimulated prior to infection and fusion, the cells were seeded in 6 well plates at 1x10⁶ cells per well. Stimulants were added at the appropriate concentration, and incubated at 37°C for 18-24 hours. The agents used for stimulation were lipopolysaccharide (LPS) at 1mg/mL, phorbol 12-myristate 13-acetate (PMA) at 200ng/mL (both from Sigma-Aldrich, Dorset, UK) and interferon- γ (IFN γ) at 10ng/mL (Invitrogen, Paisley, UK).

2.3.2 Infection of THP-1 cells

20mL peripheral blood was taken from patients with chronic HCV. All patients gave written informed consent to participate, and the protocol was approved by Moorfields and Whittington Research Ethics Committee (reference 09/H0721/25). The blood was allowed to clot for at least 20 minutes and then centrifuged at 1500rpm for 10 minutes. The serum layer was removed and stored in aliquots at -80°C until use. Viral load was available from the clinical record of all patients, and was measured using the Roche Cobas Amplicor.

Where THP-1 cells had been pre-stimulated, the vast majority of cells were adherent. Medium and non-adherent cells were removed, and the adherent cells were washed 3 times with PBS. The medium was replaced with RPMI plus 2% FCS. Unstimulated THP-1 cells in suspension were pelleted by centrifugation at 1200rpm for 5 minutes then resuspended in RPMI plus 2% FCS. The cells were seeded at a density of 10^6 cells/mL. Serum from patients with chronic HCV infection was added to the cells at a ratio of 1 viral copy per cell. Where HCVcc was used, this was added at a ratio of 10 viral copies/cell. Cells were incubated with virus at 37°C overnight.

After this incubation period, the medium containing virus was removed and the cells were washed 3 times to remove non-bound virions. Adherent pre-stimulated THP-1 cells were washed in the flask, whilst suspension cells were transferred to a 15mL Falcon tube then centrifuged at 1500rpm for 5 minutes and resuspended in fresh RPMI for each wash. Adherent cells were removed from the flask using a rubber cell scraper and transferred to a 15mL Falcon tube for fusion.

2.3.3 Cell fusion

The cell fusion protocol using primary human monocytes was optimised by Dr Alia Javaid. An identical fusion protocol was followed whether primary monocytes or THP-1 cells were used.

Huh7.5 cells were trypsinised and residual trypsin removed by washing once in DMEM. They were counted, and mixed with the monocytes at a 1:1 ratio. The cells were pelleted together by centrifugation at 1500rpm for 5 minutes. The supernatant was removed completely before resuspension of the cell mix in pre-warmed polyethylene glycol 1500 (PEG-1500; Roche Diagnostics, Burgess Hill, UK), 250 μ L per 2×10^6 total cells. PEG-1500 was added slowly to the cells over 1 minute with gentle stirring, before incubation at 37°C for 2 minutes. 10mL pre-warmed DMEM with additives (10% FCS/antibiotics) was slowly added over 3 minutes with gentle stirring to dilute the PEG. After a further 5 minute incubation at 37°C, the cells were pelleted by gentle centrifugation (1200rpm for 5 minutes), before resuspension in DMEM plus additives. The cells were seeded in 6 well plates at a density of 2×10^5 cells per well (when THP-1 cells were used for fusion) or 5×10^5 cells per well (when primary monocytes were used). The fused cells were maintained at 37°C for up to 7 days, with RNA extraction for HCV RNA quantification at various time points.

2.4 Drug inhibition assays

After fusion, the cells were left to settle overnight. The following day, inhibitory drugs were added to each well at a range of concentrations. Drugs were diluted in drug dilution medium (RPMI plus 2% FCS and 0.5% DMSO). Control wells with

no active drug were treated with drug dilution medium only. The plates were maintained at 37°C for up to 5 days, with RNA extraction for HCV RNA quantification at various time points. Where the cells were maintained for more than 3 days, the medium was removed at day 3 and replaced with fresh growth medium and drug.

2.4.1 Antiviral drugs

The antiviral agents used were interferon- α 2a (Cambridge Bioscience, Cambridge, UK), ribavirin (Sigma-Aldrich), telaprevir (kind gift from Janssen Virology), alisporivir (kind gift from Novartis Pharma, Switzerland) and SB 9200 (supplied by Spring Bank Pharmaceuticals, Milford, MA). The range of concentrations used was 10-100 IU/mL (IFN- α 2a); 5-50 IU/mL (ribavirin); 0.05-1.0 μ M (telaprevir and alisporivir) and 0.01-10 μ M (SB 9200).

2.4.2 Cytokines and stimulants

Where cytokines/stimulants were added to fused cells, this was done immediately after cell fusion. Agents used were osteopontin (PeproTech, London, UK) and SAG (Enzo Life Sciences (UK) Ltd, Exeter, UK).

2.4.3 Cell viability assay

Cell viability was measured after incubation with antiviral drugs to exclude significant drug toxicity at the drug concentrations used. An ATP quantification assay was used (CellTiter-Glo; Promega, Southampton, UK). Fused cells were seeded into opaque 96 well plates and left overnight. Antiviral drugs or drug dilution medium were then added, with each drug concentration tested in

quadruplicate. Media and drugs were refreshed at day 3. On day 5 after fusion, the plates were brought to room temperature and plain medium (with no cells) was applied to the control wells. An equal volume of CellTiter-Glo Reagent was added to each well and gently mixed by swirling for 2 minutes. The plates were incubated at room temperature for 10 minutes before the luminescence signal was read on a BMG FLUOstar Optima plate reader.

2.5 Concentration of viral particles on a sucrose gradient

To establish if infectious viral particles were released during replication in the capture-fusion system, supernatants were collected from non-drug treated wells of two capture-fusion experiments (1 with serum from a HCV G1 donor and 1 with a G3 donor). In each experiment, a total of 12 mL supernatant was collected from non-drug treated wells at day 5 post fusion. Cell debris were removed by filtration through a 0.45µm filter, then 10 mL was layered on 4 mL 20% sucrose. Supernatants were centrifuged (24,000 x g) for 2 hours and the pellet resuspended in 1mL RPMI. For capture-fusion experiments using concentrated supernatant, 1×10^6 prestimulated THP-1 cells were incubated with 1 mL of concentrated supernatant for 24 hours before fusion.

2.6 Molecular biology techniques

2.6.1 RNA extraction from cells using TRIzol reagent

For RNA extraction from adherent cells, the supernatant was removed from each well and 1mL TRIzol reagent (Invitrogen) added to the adherent cells. The cellular proteins and nucleic acids were dissolved in TRIzol by repeated pipetting, then transferred to a 1.5mL Eppendorf tube and stored at -80°C prior to RNA

purification. In certain experiments, supernatants removed from the cells were filtered and stored at -80°C for subsequent HCV RNA detection and use in re-infection experiments.

For RNA extraction from suspension cells (primary monocytes, unstimulated THP-1 cells and fused cells immediately following fusion), an aliquot containing 5×10^5 cells was centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 1 mL TRIzol reagent, with repeated pipetting, before transfer to a 1.5mL Eppendorf tube and storage at -80°C.

2.6.2 RNA purification

Frozen samples of RNA in TRIzol reagent were thawed at room temperature to allow dissociation of the nucleoprotein complexes. 200µL chloroform was added to each sample and mixed by shaking. After 5 minutes at room temperature, the samples were centrifuged at 12,000rpm at 4°C, for 15 minutes. 400µL of the upper (aqueous) phase containing RNA was transferred to a clean Eppendorf tube. 500µL ice-cold isopropanol was added to each, and mixed by inverting. The samples were incubated for 10 minutes at -20°C before centrifugation at 12,000rpm at 4°C, for 15 minutes. The supernatant was discarded and 1mL cold 70% ethanol added to each tube to wash the RNA pellet. After centrifugation at 7500rpm for 10 minutes at 4°C, the ethanol was removed and the RNA pellet allowed to air dry, before resuspension in nuclease-free water. The samples were heated to 65°C for 5 minutes to aid dissolution. RNA per sample was then quantified using a Nanodrop spectrophotometer.

2.6.3 DNase treatment of purified RNA

If necessary, purified RNA was further diluted in nuclease-free water to a concentration of less than 500ng/μL. RNA was then combined with DNase and 10x DNase buffer (Promega) in the following quantities per sample:

2μL RNA

1μL DNase

1μL 10x DNase buffer

6μL nuclease-free water

Each 10μL reaction was incubated at 37°C for 30 minutes. 1μL stop solution was added to each, followed by incubation at 65°C for 10 minutes before being placed back on ice.

2.6.4 Quantification of total RNA by the RiboGreen assay

Spectrophotometric estimation of RNA yield can be inaccurate due to presence of even trace amounts of contaminants such as DNA, phenol or protein. Where the final measurement of HCV RNA is to be normalised against total RNA extracted from the sample, a more accurate method should be used for quantification of total RNA, such as the RiboGreen assay (Invitrogen). RNA is DNase treated prior to measurement, as the RiboGreen dye will bind both RNA and DNA, thus falsely elevating results in untreated samples.

RNA taken from the 10ul DNase reaction was diluted 1in 100 in TE buffer, sufficient to allow each RNA sample to be measured in duplicate. 100μL of each diluted RNA was transferred to a white 96 well plate, in duplicate. The manufacturer's RNA standard was diluted to provide a standard curve ranging

from 20ng/mL to 1000ng/mL, and was also added to the plate in duplicate. 100µL TE buffer was used to measure background fluorescence. RiboGreen dye was diluted 1/200 in TE buffer and 100µL added to each well of RNA samples and standards. The plate was incubated for 5 minutes in the dark before being read on a BMG FLUOstar Optima plate reader. The average of the background values was subtracted from all other measurements. Values generated from the diluted standard were used to construct a standard curve using Prism software (GraphPad, La Jolla, CA). RNA concentrations for each sample were interpolated from the standard curve.

2.6.5 Extraction of viral RNA from supernatants

Viral RNA was extracted from supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Crawley, UK). 280µL of supernatant was thoroughly mixed with 1.12mL Buffer AVL (premixed with 11.2µL carrier RNA) and left at room temperature for 10 minutes. 1.12mL 100% ethanol was added and vortexed to mix. The sample was applied to a QIAamp Mini column, 600µL at a time, and centrifuged at 8000rpm for 1 minute until all the sample had been passed through the column. The filtrate was discarded after each centrifugation. 500µL of Buffer AW1 was added to the column and centrifuged at 8000rpm for 1 minute. The filtrate was discarded, and 500µL of Buffer AW2 was added to the column before centrifugation at 14000rpm for 3 minutes. The filtrate was discarded and the column centrifuged again at 14000rpm for 1 minute to remove any residual Buffer AW2. The column was transferred to a 1.5mL Eppendorf tube and 60µL Buffer AVE added. After 1 minute incubation at room temperature, the column and tube were centrifuged at 8000rpm for 1 minute to elute the RNA. Due to the presence

of carrier RNA, the RNA obtained was not quantified by spectrophotometer. RNA was stored at -80°C.

2.6.6 Production of an RNA standard for absolute quantification of HCV RNA

In order to measure HCV RNA quantitatively by PCR, a standard of known copy number is required to generate a standard curve. An RNA standard corresponding to an area of the viral genome slightly larger than the region amplified during the quantitative RT-PCR reaction was chosen to act as a standard in these experiments. An RNA standard was chosen over a PCR product DNA standard as there were theoretical concerns that a DNA standard may underestimate sample copy number as the PCR reaction efficiency for the DNA standard may be higher than that of sample, due to lack of the reverse transcription step, relatively small product size and lack of secondary structure. Use of an RNA standard addresses at least some of these concerns.

To generate the RNA standard, a plasmid containing DNA reverse transcribed from the JFH-1 genome was kindly supplied by Dr John McLauchlan (University of Glasgow Centre for Virus Research, Glasgow, UK).

To isolate the JFH-1 DNA, the plasmid was digested using the restriction endonuclease Xba1 (New England Biolabs). The reaction mix comprised:

20µg plasmid DNA

3µL Xba1

5µL 10x endonuclease buffer

Nuclease-free water to a final volume of 50 μ L.

The reaction was incubated at 37°C for 2 hours.

The DNA was then cleaned up using a phenol/chloroform extraction step. The reaction volume was adjusted to 100 μ L with water. 100 μ L phenol/chloroform was added, and centrifuged at 12000rpm for 1 minute. The upper aqueous layer was transferred to a fresh Eppendorf tube and 100 μ L chloroform added. After mixing, the reaction was again centrifuged at 12000rpm for 1 minute. The upper aqueous layer was transferred into a fresh Eppendorf tube. The DNA was precipitated by adding 0.3M sodium acetate and 250 μ L 100% ethanol then incubating at -20°C for at least 10 minutes. DNA was pelleted by centrifugation for 2 minutes at 12000rpm, then washed in 70% ethanol. After another 2 minute centrifugation at 12000rpm the supernatant was discarded and the pellet air-dried then resuspended in 52 μ L water.

To convert the ends of the excised JFH-1 DNA from sticky to blunt, to facilitate transcription, it was treated with mung bean nuclease (New England Biolabs).

The reaction mix contained:

52 μ L linearised, clean DNA

6 μ L 10x Buffer

2 μ L mung bean nuclease

This reaction was incubated at 30°C for 30 minutes before a further phenol/chloroform clean up step, which was performed as described above. The DNA pellet was resuspended in 20 μ L nuclease-free water and quantified using a Nanodrop spectrophotometer.

The excised JFH-1 DNA contains a RNA polymerase promoter sequence which was used to initiate transcription, using the T7 Megascript Kit (Ambion, Paisley, UK).

A reaction mix was made up as follows:

1µg linearised, mung bean nuclease treated, clean DNA

2µL ATP

2µL CTP

2µL GTP

2µL UTP

2µL 2x Reaction Buffer

2µL T7 enzyme mix

Nuclease-free water to a final volume of 20µL

The mixture was gently mixed by flicking and incubated at 37°C for 3 hours. 2µL of the reaction product was run on a 1% agarose gel containing GelRed to ensure the presence and integrity of the transcribed RNA.

The RNA product was treated with TurboDNase for 15min at 37 °C, then 15 µL ammonium acetate and 115 µL nuclease free water were added to the reaction mix. A further phenol/chloroform clean-up step was performed, as described above. The RNA pellet was resuspended in 50 µL nuclease free water and quantified using the RiboGreen assay (as described in Section 2.6.4). The approximate number of RNA copies in the sample was calculated as follows:

$$\text{Copies}/\mu\text{L} = (\text{total RNA (ng}/\mu\text{L)})/340 \times \text{JFH-1 genome (bases)} \times 6.022 \times 10^{23}$$

Based on published literature, the length of the JFH-1 genome was taken as 9678 bases [131]. Once RNA copies/ μL in the sample was calculated, the undiluted standard stock was stored at $-80\text{ }^{\circ}\text{C}$. The RNA standard was diluted to a concentration of 2×10^9 copies/ μL in Qiagen nucleic acid buffer and stored in $10\mu\text{L}$ aliquots at $-80\text{ }^{\circ}\text{C}$ which were used to make fresh serial dilutions for each PCR experiment.

2.6.7 RTqPCR measurement of HCV RNA

HCV RNA was quantified using the QuantiTect Virus Kit (Qiagen) and a TaqMan HCV-specific primer and probe set (Applied Biosystems, Paisley, UK). Serial dilutions of the RNA standard, described above, amplified in this assay reproducibly generated a standard curve ranging from $10 - 10^7$ copies, with an efficiency of close to 100%. Unfortunately, no data were available calibrating this assay against the WHO HCV RNA reference standard, so sensitivity limits could not be expressed in IU/mL [266].

Samples were set up for this combined reverse transcription-qPCR assay on ice as follows:

25-50ng RNA template

4 μL 5x QuantiTect Virus NR MasterMix

0.2 μL 100x QuantiTect virus RT mix

1 μL primer/probe

RNase-free water to final volume 20 μL

The samples were run on a RotorGene 3000 real time cycler using the following cycling conditions:

Reverse transcription: 50°C for 20 minutes

Activation of hot-start Taq polymerase: 95°C for 5 minutes

Cycling: 95°C for 15 seconds/60°C for 45 seconds, for 45 cycles.

Serial dilutions of the HCV IRES PCR product and no-template negative controls were included in all experiments. HCV RNA was quantified by extrapolation from the standard curve, and normalised to total RNA present in the sample.

2.6.8 TOPO cloning and sequencing of HCV RNA

The 5'UTR of HCV RNA obtained from fusion experiments using serum from HCV genotype 1a infected donors was amplified, cloned and sequenced to confirm alignment with HCV genotype 1a reference sequence H77, and not with the genotype 2 high replicating laboratory strain JFH-1.

For cDNA synthesis, purified RNA was obtained as described above. 1µg of each RNA sample was treated with 1µL DNase (Promega) and an appropriate volume of 10x DNase buffer and water and incubated at 37°C for 30 minutes. 16µL of DNase-treated RNA was then mixed with 4µL SuperScript VILO MasterMix (Life Technologies, Paisley, UK) and run on a thermal cycler under the following conditions:

25°C for 10 minutes/42°C for 60 minutes/85°C for 5 minutes.

cDNA was stored at -20°C until use.

The Expand High Fidelity PCR system (Roche Diagnostics) was used to produce a blunt-ended PCR product for subsequent cloning. Mix 1 and Mix 2 were prepared separately.

Mix 1:

0.5 μ L dNTP (0.25mM)

1.5 μ L forward primer

1.5 μ L reverse primer

2 μ L cDNA

RNase-free water to final volume 25 μ L

Mix 2:

0.75 μ L polymerase enzyme mix

5 μ L Buffer without Mg

5 μ L Mg (final concentration 2.5mM)

RNase-free water to final volume 25 μ L

Primers used were:

Forward: 5'-AGCGTCTAGCCATGGCGT-3'

Reverse: 5'-GCACGGTCTACGAGACCT-3'

Mix 1 and Mix 2 were combined on ice run on a RotorGene 3000 real time cycler using the following cycling conditions:

Activation: 95°C for 15 minutes

Cycling: 95°C for 15 sec/58°C for 30 sec/72°C for 2 min for 45 cycles

Extension: 72°C for 7 minutes.

The products were stored at -20°C until purification and concentration, which was performed using the Qiagen MinElute PCR Purification kit (Qiagen). 250 µL Buffer PB was added to each PCR reaction and mixed. Each sample was applied to a MinElute column in a 2mL collection tube and centrifuged at 13,000rpm for 1 minute. The flow-through was discarded and the sample washed with 750 µL Buffer PE and centrifuged at 13,000rpm for 1 minute. The flow-through was discarded again and the sample centrifuged at 14,200rpm for 1 minute. The column was placed in a clean 1.5 mL microcentrifuge tube and 10 µL Buffer EB applied. The column was left to stand for 1 minute then centrifuged at 13,000rpm for 1 minute.

Cloning was performed using the ZeroBlunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). The cloning reaction was prepared by combining the following for each PCR sample:

4 µL PCR product

1µL salt solution

1µL TOPO vector.

The cloning reaction mixture was incubated at room temperature for 30 minutes and then placed on ice. For transformation, 100 µL chemically competent *E. coli* (DH5αTM-T1^R) were thawed on ice for each transformation reaction. Each cloning reaction mixture was added to an aliquot of cells, mixed gently and incubated on ice for 30 minutes. The cells were then heat-shocked on a heat block at 42°C for 30 seconds and immediately placed back on ice. 250µL S.O.C. medium at room

temperature was added to each sample, then incubated at 37°C for 1 hour with horizontal shaking (200rpm). 50µL from each transformation was spread onto a pre-warmed agar plate containing ampicillin (50µg/mL). The plates were incubated at 37°C overnight. The following day, 2 colonies from each plate were transferred into 5mL LB Broth containing ampicillin (50µg/mL). The tubes were incubated at 37°C overnight with horizontal shaking (200rpm).

For each sample, DNA was isolated and purified from the total volume of overnight culture using the PureLink HighPure Plasmid MiniPrep Kit (Invitrogen). The cells were isolated by centrifugation at 4000xg for 10 minutes and the medium removed. 0.4mL of resuspension buffer with RNaseA was added, and the cells were resuspended until the mixture was homogenous. 0.4mL lysis buffer was added, the mixture inverted, and left to stand for 5 minutes. 0.4mL precipitation buffer was added and mixed by inverting the tube until the sample appeared homogenous. The lysate was centrifuged at 13,000xg for 10 minutes at room temperature. The supernatant was transferred to a column, previously equilibrated with 2 mL equilibration buffer. The column was washed twice with 2.5 mL wash buffer. 0.9 mL elution buffer was applied to the column and the DNA eluted into a clean microcentrifuge tube. 0.63 mL isopropanol was added to the tube, mixed well, and centrifuged at 12,000xg for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet resuspended in 1 mL 70% ethanol. The tube was centrifuged again at 12,000xg for 5 minutes at 4°C, the supernatant discarded, and the pellet left to air dry for 10 minutes before resuspension in 30 µL TE buffer. DNA in each sample was quantified using a

Nanodrop spectrophotometer and the samples were stored at -20 °C until further use.

To confirm the presence of a correctly sized (265bp) product in each cloned plasmid DNA sample, an EcoR1 digest was performed (New England Biolabs, Hitchin, UK). For each sample, the following reaction mixture was prepared:

1 µg purified plasmid DNA

1 µL EcoR1

2µL EcoR1 Buffer

RNase-free water to final volume 20µL.

This mixture was incubated at 37°C for 2 hours before 5 µL was run on a 2% agarose gel alongside a 100bp ladder.

It was not possible to check the orientation of the PCR product within the plasmid by restriction enzyme analysis as no suitable single cut site in the plasmid and product could be identified. Therefore each sample containing an appropriately-sized insert on Eco-R1 digestion was sent for sequencing (performed by Source Bioscience, Nottingham, UK) using both T3 and T7 stock primers. Sequences obtained were aligned with the 5'UTR of the G1a reference sequence H77 and JFH-1 using BioEdit Sequence Alignment Editor software [267].

2.6.9 Quantification of mRNA expression

For analysis of host gene expression by mRNA quantification, cDNA was synthesised from purified, DNase-treated sample RNA, as described above. 1 µL oligodTs and 1 µL random hexamers (Promega) were added to each sample of

10 μ L DNase-treated RNA and heated at 65°C for 5 minutes before being placed on ice.

For reverse transcription, the following mix was prepared (using Promega RT reagents) and added to each sample:

14 μ L RT buffer

2 μ L dNTP (10mM)

1 μ L RNAsin

1 μ L MMLV reverse transcriptase

6 μ L RNase-free water.

The reaction was incubated at 42°C for 1 hour, then 75°C for 10 minutes and cDNA product stored at -20°C for subsequent PCR.

mRNA quantification was performed by real-time PCR using the QuantiTect-SYBR-Green real time PCR system (Qiagen). cDNA was diluted 1/10 in nuclease free water.

For each PCR, the following reaction mix was set up:

12.5 μ L 2x QuantiTect SYBR-Green PCR MasterMix

0.5 μ L Forward Primer (10 μ M)

0.5 μ L Reverse Primer (10 μ M)

2 μ L diluted cDNA

RNase free water to final volume 25 μ L.

PCR reactions were run in duplicate for each sample. Cycling conditions were:

Activation: 95°C for 15 minutes

Cycling: 94°C for 15 sec (denaturation)/60°C for 30 sec (annealing)/72°C for 30 sec (extension) for 45 cycles.

Primers used were:

MxA: Forward: 5'-GTTGGAGGCACTGTCAGGAGTTGC-3'
Reverse: 5'-CTACCTCTGAAGCATCCGAAATCTC-3'

CD81: Forward: 5'-AAGCAGTTCTATGACCAGGCCCTAC-3'
Reverse: 5'-TGAGGTGGTCAAAGCAGTCAGTG-3'

SR-B1: Forward: 5'-ATGAAATCTGTGCGCAGGCATTG-3'
Reverse: 5'-TGCATCACCTTGGGCATCA-3'

CD32: Forward: 5'-TTCAAGGCCAACAACAATGA-3'
Reverse: 5'-GGAGAAGGTGGGATCCAAAT-3'

CD64: Forward: 5'-GTGTCATGCGTGGAAGGATA-3'
Reverse: 5'-GCACTGGAGCTGGAAATA-3'

CD16: Forward: 5'-ACAGGTGCCAGACAAACCTC-3'
Reverse: 5'-TTCCAGCTGTGACACCTCAG-3'

Claudin-1: Forward: 5'-GCATGAAGTGTATGAAGTGCTTGGA-3'
Reverse: 5'-CGATTCTATTGCCATACCATGCTG-3'

Occludin: Forward: 5'-AAGAGTTGACAGTCCCATGGCATA-3'
Reverse: 5'-ATCCACAGGCGAAGTTAATGGAAG-3'

LDL-R: Forward: 5'-CAACGGCTCAGACGAGCAAG-3'
Reverse: 5'-AGTCACAGACGAACTGCCGAGA-3'

GAPDH: Forward and reverse primers obtained from PrimerDesign (Southampton, UK). Sequences not available.

Data acquisition was performed during the extension step. Melt curve analysis was performed to exclude mispriming. Results were normalised to expression of GAPDH, measured in the same PCR run, and expressed as relative gene expression using the $2^{-\Delta\Delta CT}$ method.

2.5.10 Confirmation of stability of GAPDH expression

In this project, cellular mRNA expression was measured in 2 separate series of experiments - quantification of interferon stimuable gene expression in fusion experiments, and quantification of receptor expression by stimulated THP-1 cells. Stability of reference gene expression for normalisation of mRNA quantification was tested in both experimental conditions. The geNorm Plus kit (PrimerDesign) was used, which tested the stability of a panel of 12 reference genes (beta actin, GAPDH, VIPAR, ERCC6, UBE2D2, UBE4A, PRDM4, ENOX2, SCLY, TWY1, RNF20 and 18S rRNA). To test reference gene stability in fusion experiments, cDNA was synthesised (as described above) from RNA extracted from unfused Huh7.5 cells and from RNA extracted at 5 days after fusion from fused cells treated with drug dilution medium or telaprevir at a range of concentrations. For THP-1 stimulation experiments, cDNA was synthesised from RNA extracted from unstimulated THP-1 cells, and 2, 6, 12 and 24 hours after treatment with PMA (200ng/mL) and IFN γ (10ng/mL). In each PCR run, expression of 4 reference genes was measured from 8 experimental samples, each in duplicate. cDNA from the same experimental samples was used in the 3 PCR runs required to measure expression of all 12 reference genes. Results were analysed using qbase+ software (Biogazelle, Zwijnaarde, Belgium). Considering both series of

experiments, stability of GAPDH expression was considered acceptable for use as a reference gene for normalisation in these experiments (Figure 2-1).

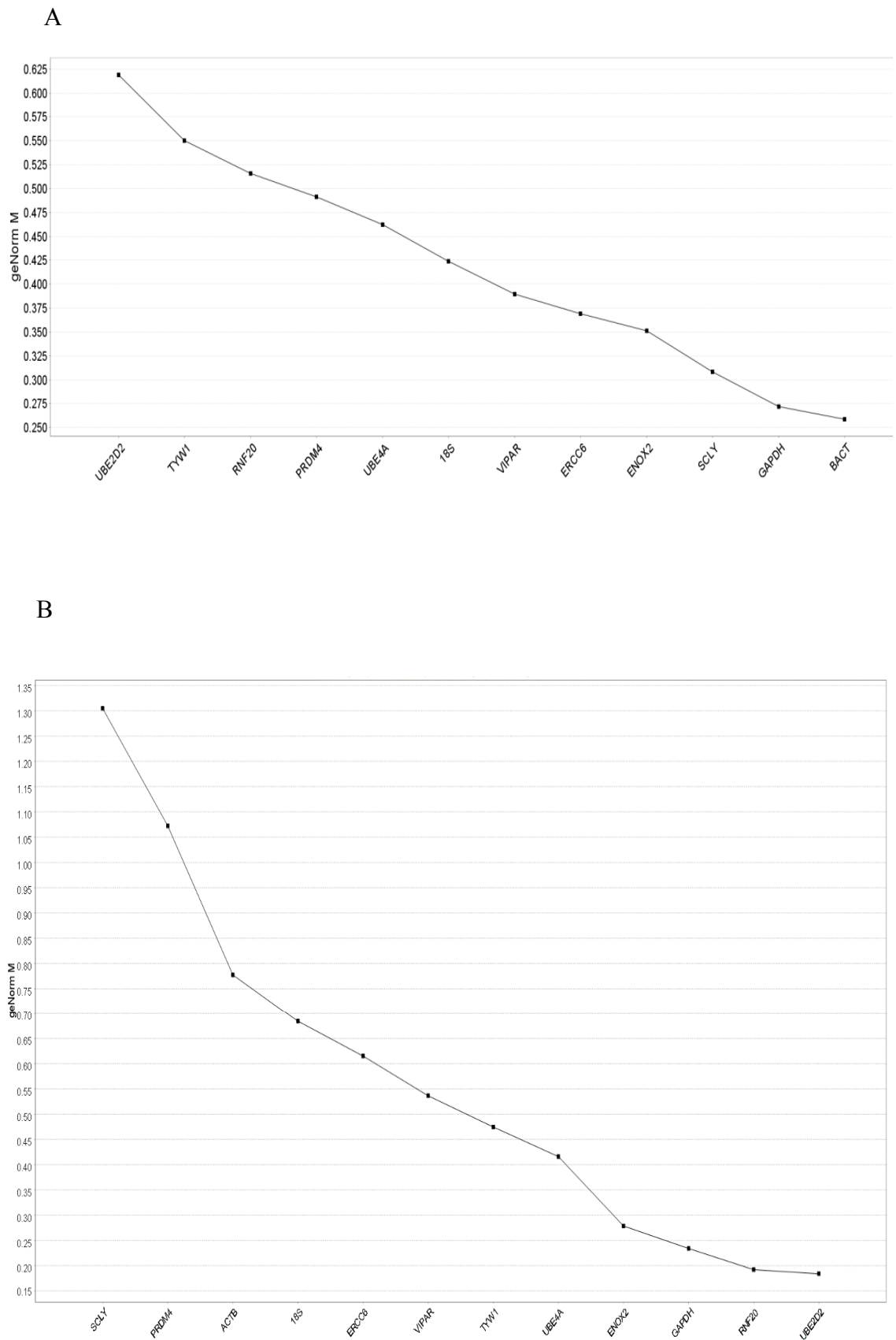


Figure 2-1. Average relative expression stability of reference genes. Of the panel of 12 reference genes tested, relative stability in capture-fusion experiments (A) or stimulation of THP-1 cells (B) was measured using the geNorm assay. The lower the geNorm M value, the greater relative stability in the experimental samples tested (calculated according to methods described by Hellemans *et al* 2007 [268]).

2.7 Generation of cell culture produced HCV (HCVcc)

JFH-1 is a unique strain of HCV which can replicate in tissue culture, producing virions which are infectious to Huh-7.5 cells (HCVcc) [28, 99].

A plasmid containing DNA reverse transcribed from the JFH-1 genome was kindly supplied by Dr John McLauchlan (University of Glasgow Centre for Virus Research, Glasgow, UK). Digestion and clean-up of the plasmid DNA, conversion of the excised DNA ends from sticky to blunt by mung bean nuclease treatment and transcription of JFH-1 DNA was performed as described in Section 2.6.6.

2.7.1 Electroporation of transcribed JFH-1 RNA into Huh7.5 cells

10^6 Huh7.5 cells were suspended in 400 μ L PBS and transferred to an electroporation cuvette. 10 μ L transcribed RNA was added to the cuvette, and electroporated in a BioRad electroporator set to a voltage of 270V and capacitance of 950 μ F. The electroporated cells were made up to 6mL with DMEM plus additives and split between 3 wells of a 6 well plate. They were maintained at 37°C for up to 12 days. After the first 72 hours, the medium was changed every 2-3 days and supernatants stored at -80°C to provide HCVcc stocks.

2.7.2 Quantification of HCVcc by RT-qPCR

Supernatants from cells electroporated with JFH-1 RNA were filtered through 0.45 μ M sterile filters to remove any cellular debris. Viral RNA was extracted from the supernatants using the QIAamp Viral RNA Mini Kit, as described in section 2.5.5. 2 μ L of extracted, purified RNA was used as a template for in the RT-qPCR assay as described in section 2.5.7. The resulting HCV measurement was related

to the original supernatant by back-calculation and expressed as HCVcc copies/mL supernatant.

2.7.3 Measurement of HCVcc infectivity by the 50% tissue culture infectious dose (TCID₅₀) assay

Due to the high error rate intrinsic in HCV replication, a substantial number of virions produced will be defective, or poorly infective/replicative. Therefore quantification of HCVcc by PCR is likely to overestimate the number of functional viral particles. An infectivity assay is more useful to quantify the infectiousness of the HCVcc produced.

Huh7.5 cells were seeded into 96 well plates at a density of 3000 cells in 100µL per well. After overnight incubation at 37°C, 50µL of supernatant containing HCVcc was added to each well in row A of the plate. After mixing, 50µL of the row A supernatant was transferred to row B, and so on to make serial 1 in 3 dilutions of HCVcc down the plate. The plate was incubated at 37°C for 72 hours, then the cells fixed by removal of supernatant and submersion of the plate in ice-cold methanol for 20 minutes. For immunofluorescence staining, the fixed cells were washed 3 times by submersion of the plate in PBS. Primary antibody (sheep anti-NS5A) was diluted 1/5000 in PBS/1% FCS and 50µL added to each well for 1 hour at room temperature. After 3 further washes in PBS, 50µL of secondary antibody (AlexaFluor 488 donkey anti-sheep, diluted 1/500 in PBS/1% FCS) was added per well and incubated in the dark at room temperature for one hour. The cells were washed in PBS 3 times. 50µL water was added per well for storage at 4°C in the dark until the cells were imaged.

For calculation of the TCID₅₀, the plate was visualised using an Olympus Timelapse fluorescence microscope at 5x magnification. The number of wells with foci of HCV infection at each dilution of input supernatant were enumerated, and used to calculate the TCID₅₀ using the formula devised by Reed and Muench [269].

2.7.4 HCV quantification in Huh7-J20 cells

Huh7-J20 cells stably express a fusion protein comprising enhanced green fluorescent protein and secreted alkaline phosphatase (SEAP), linked by the HCV NS4A/NS4B cleavage site. In the presence of sufficient HCV replication, the enzymatic action of the HCV NS3/4A protease acts on the cleavage site to release SEAP into the supernatant. To quantify replication of JFH-1 using this technique, Huh7-J20 cells were seeded into a 96 well tissue culture plate at a density of 1×10^3 cells per well. After overnight incubation at 37°C, the cells were infected in quadruplicate with JFH-1 at concentrations of 0.1, 0.2 or 0.3 TCID₅₀/well. The cells were then maintained at 37°C for 72 hours.

Quantification of SEAP in the cell supernatants was performed using the Great EscAPe chemiluminescent SEAP assay kit (ClonTech, Takara Bio Europe SAS, Saint-Germain-en-Laye, France). Supernatant was transferred from each well to a microcentrifuge tube and centrifuged briefly. 25 µL of each supernatant was transferred to a white 96 well plate. Each sample was tested in triplicate. Placental alkaline phosphatase (diluted 1/40) and plain medium (DMEM) were included as positive and negative controls, respectively. 75 µL 1x SEAP assay

Dilution Buffer was added to each well. The plate was sealed and incubated on a heat block for 30 minutes at 65°C. The plate was cooled on ice for a few minutes then allowed to come to room temperature. 100 µL of SEAP Substrate Solution at room temperature was added to each well and the plates incubated at room temperature for a further 30 minutes before luminescence was read on a BMG FLUOstar Optima plate reader. To quantify replication of patient-derived HCV, Huh7-J20 cells were used in cell fusion experiments in place of Huh7.5 cells. 2.5×10^5 fused cells/well were plated in triplicate per fusion in 12 well tissue culture plates. Medium was changed at day 3 and supernatants stored at -80°C. Supernatants were also taken at day 5 post-fusion, at which time RNA was extracted from the cells using TRIzol, for subsequent confirmation of HCV RNA by RT-qPCR (as described in section 2.5.7). SEAP was measured in the day 3 and day post-fusion supernatants, using the method described above.

2.8 Enzyme-linked immunosorbent assay (ELISA)

To identify HCV IgG (+), RNA (-) samples containing antibody with the greatest avidity for JFH-1, an ELISA was performed based on published methodology [270]. ELISA Immunosorb plates were coated with 50 µL/well galanthus nivalis agglutinin (5µg/mL diluted in bicarbonate buffer; Sigma-Aldrich) and incubated at 37°C for 2 hours. The wells were washed once with PBS/Tween (1 x PBS/0.05% Tween) then blocked overnight with 200 µL/well 5% milk in PBS/Tween. The following day, the plates were washed thrice with PBS/Tween. Lysed Huh7.5 cells infected with JFH-1 were diluted 1/50 in 5% milk/PBS/Tween and 50 µL applied to each well. Lysed 298T cells were included at the same concentration as a negative control. The plates were incubated at room temperature for 2 hours

before being washed thrice with PBS/Tween. Test sera were diluted 1/50 in 5% milk/PBS/Tween and 50 μ L applied to each well. Each serum was tested in triplicate, and known HCV IgG (-) serum was included as a negative control. The plates were incubated at room temperature for 2 hours before being washed 5 times with PBS/Tween. AP labelled goat anti-human IgG (Life Technologies) was diluted 1/500 in 5% milk/PBS/Tween and 50 μ L added to each well. The plates were incubated at room temperature for 2 hours before being washed 5 times with PBS/Tween. 50 μ L p-nitrophenyl phosphate substrate (Sigma-Aldrich) was added to each well, then incubated in the dark at room temperature for 30 minutes before luminescence was read at 405nm. The samples with greatest luminescence (and therefore greatest titre of antibodies to JFH-1 proteins) were used in subsequent experiments investigating antibody-mediated enhancement of JFH-1 entry into monocytes.

2.9 Immunofluorescence

Stimulated THP-1 cells or fused cells for immunofluorescence were seeded onto coverslips and grown for 3 to 5 days before fixation in 4% paraformaldehyde (PFA). After fixation, the cells were washed with PBS and permeabilised using PBS/0.2% Triton for 30 minutes at room temperature. They were washed again in PBS prior to blocking in PBS/10% FCS for 30 minutes at room temperature. After a further wash in PBS, primary antibody diluted in PBS/10% FCS was added for one hour at room temperature. Unbound primary antibody was removed by 3 washes with PBS/0.05% Tween, each lasting 10 minutes. Secondary antibody diluted in PBS/10%FCS was then added for an hour in the dark at room temperature, followed by 3 further washes in PBS/0.05% Tween. The process

was repeated for immunostaining of a second target. When staining for HCV non-structural proteins, replicon cells and uninfected Huh-7.5 cells were included as positive and negative controls.

After staining, the coverslips were mounted on microscope slides using a drop of Vectashield mounting medium (Vector Laboratories Ltd, Peterborough, UK). The slides were stored at -20°C overnight before imaging on an Olympus MM Leica Fluorescence microscope at 60x magnification.

2.9.1 Antibodies used for immunofluorescence

The primary antibodies used were sheep anti-NS5A (used at 1/5000; kind gift from Prof Mark Harris, University of Leeds) and rabbit anti-human albumin (used at 1/500; Dako UK Ltd, Ely, UK). The secondary antibodies used were AlexaFluor 488 donkey anti-sheep (used at 1/500) and AlexaFluor 568 donkey anti-rabbit (used at 1/500; both from Life Technologies).

2.10 Flow cytometry and fluorescence-activated cell sorting (FACS)

2.10.1 Flow cytometry

Flow cytometry was used to quantify receptor expression on stimulated and unstimulated THP-1 cells. THP-1 cells were incubated for 24 hours in medium alone (RPMI/2% FCS), medium plus PMA 200ng/mL, or medium plus PMA 200ng/mL and IFN γ 10ng/mL. The cells were washed in PBS and adherent cells removed using a cell scraper. Viable cells were identified by trypan blue staining and 0.5×10^6 cells from each group transferred to each of 10 FACS tubes. The tubes were centrifuged at 1700rpm for 5 minutes, the supernatant discarded and

the cells resuspended in 1 mL ice-cold FACS buffer (PBS/5% FCS/0.5% sodium azide/0.9% EDTA). The cells were centrifuged again at 1700rpm for 5 minutes, the supernatant discarded, and the cells resuspended in 100 μ L FACS buffer and incubated on ice for 1 hour. Appropriate primary antibody or isotype control was added to each tube, diluted where necessary in PBS/3% BSA. The tubes were incubated at 4°C in the dark for 45 minutes. FACS buffer was added to each tube to a volume of 1mL and the tubes centrifuged at 1700rpm for 5 minutes. The supernatants were discarded and this wash step repeated twice more. The cells were resuspended in 100 μ L FACS buffer and appropriate secondary antibody added to each tube, diluted in PSA/3% BSA where necessary. The tubes were incubated at 4°C in the dark for 30 minutes. Again, FACS buffer was added to each tube to a volume of 1mL and the tubes centrifuged at 1700rpm for 5 minutes. The supernatants were discarded and this wash step repeated twice more. 300 μ L 2% paraformaldehyde (PFA) was added to each tube and stored at 4°C in the dark until analysis.

Flow cytometry was performed using a BD FACS Canto II Flow Cytometer, in conjunction with Mr Gary Warnes (Blizard Institute Flow Cytometry Core Facility). Compensation limits were set with CompBeads (BD Biosciences, Oxford, UK) stained using the same protocol.

2.10.2 Fluorescence activated cell sorting (FACS)

FACS was employed in two separate experiments; firstly to separate CD14+, CD16- and CD14+ CD16+ monocyte subsets from infected patients for

subsequent fusion experiments, and secondly to isolate fused THP-1/Huh7.5 from unfused cells prior to infection with JFH-1.

To separate monocyte subsets, total PBMCs were obtained from an HCV-infected patient, as described above. Cells were counted and transferred to a FACS tube, centrifuged at 1700rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 mL FACS buffer. This wash step was repeated twice. After the final wash, the cells were resuspended in 100 μ L FACS buffer and fluorescence-conjugated primary antibodies were added. After mixing, the cells were incubated at 4°C in the dark for 20 minutes. FACS buffer was added to final volume 1mL and the cells were centrifuged at 1700rpm for 5 minutes. The supernatant was discarded and this wash step was repeated twice. The cells were resuspended in 500 μ L FACS buffer and sorted using a BD FACSAria IIIu Cell Sorter, in conjunction with Mr Gary Warnes (Blizard Institute Flow Cytometry Core Facility). Compensation limits were set with CompBeads (BD Biosciences) stained using the same protocol.

To separate fused from unfused cells, 5×10^6 THP-1 cells and 5×10^6 Huh7.5 cells were suspended in RPMI at a density of 10^6 cells/mL and incubated with the fluorescent membrane dyes Dil (THP-1) or DiO (Huh7.5; both from Life Technologies) at 2.5 μ L/mL, at 37°C in the dark for 20 minutes. The cells were centrifuged at 1200rpm for 5 minutes. After discarding the supernatant, the cells were resuspended in RPMI and this wash step repeated twice. 0.5×10^6 of each cell type were transferred to fresh FACS tubes in 0.5mL RPMI and used to set compensation limits for subsequent cell sorting. The remaining cells were fused

using the protocol described above. The fused cells were resuspended in 1mL RPMI/10%FCS and sorted, as described above, according to single or dual fluorescence.

2.10.3 Antibodies used for flow cytometry/FACS

The primary antibodies used were mouse anti-human CD81 (1.0µg/10⁶ cells; BD Biosciences), mouse anti-human CD64 [10.1] (1.0µg/10⁶ cells), mouse anti-human CD32 [AT10] (1.0µg/10⁶ cells; both from Abcam, Cambridge, UK) and rabbit anti-human SR-B1 (1/100; Novus Biologicals, Cambridge, UK). Isotype controls were mouse IgG1, k isotype control (1.0µg/10⁶ cells; Biolegend, London, UK), rabbit IgG isotype control (1/100; Novus Biologicals) and PerCP-Cy5.5-conjugated mouse IgG1 (20 µL/10⁶ cells; BD Biosciences). Secondary antibodies were FITC rat anti-mouse IgG (1.0µg/10⁶ cells) and DyLight 488 Donkey anti-rabbit IgG (1.0µg/10⁶ cells; both from Biolegend). Fluorescence-conjugated primary antibodies were PerCP-Cy5.5-conjugated mouse anti-human CD16 and FITC-conjugated mouse anti-human CD14 (both used at 20 µL/10⁶ cells; BD Biosciences).

2.11 Statistical analyses

Data were tested for normality using the D'Agostino and Pearson test. Analyses for statistical significance used Student *t*-test for parametric data or Mann-Whitney U-test for non-parametric data. Analyses of correlation used Pearson *r* correlation coefficient for parametric data or Spearman rank correlation test for non-parametric data. In all cases, *p* < 0.05 was considered statistically significant.

3.0 Results: Replication of HCV in fused patient monocytes

3.1 Introduction

Many groups have reported that HCV associates with PBMCs in patients with chronic HCV infection, including monocytes, however whether this virus is viable, or replicates in these cells, has remained controversial [112, 116, 118, 132, 138, 271-275]. At least some of this controversy stems from technical challenges inherent in detecting low level viral replication with techniques such as negative strand PCR and immunofluorescence (reviewed in [108]). We hypothesised that detectable viral replication would occur if monocyte-associated HCV was transferred to a more permissive environment. The Huh7.5 cell line was chosen as this is highly permissive to the HCV replicon and cell culture HCV strains [276, 277]. Polyethylene glycol (PEG) causes fusion of cell membranes by exclusion of water molecules and is widely used in production of hybridomas for monoclonal antibody synthesis [278]. Previous work in our group found an increase in detectable HCV RNA over time after PEG-mediated fusion of patient monocytes with Huh7.5 cells, up to 7 days after fusion, suggesting viral replication in the fused cells. This work, performed by Dr Alia Javaid, established optimal fusion conditions which were used in fusion experiments described in this chapter (detailed in Chapter 2). This chapter describes the further development of this work, including development of a sensitive and specific PCR assay for HCV copy number quantification, fusion of monocytes infected with diverse viral genotypes and further proof of HCV replication by indirect immunofluorescence and drug inhibition. Viral replication after fusion implies presence of viable HCV in patient monocytes, and the biological significance of this as a mechanism for relapse after antiviral therapy is explored.

3.2 Development of a PCR assay for HCV quantification

Initial experiments demonstrating an increase in HCV RNA after fusion by real-time PCR used SYBR Green chemistry, a fluorescent dye which intercalates with double-stranded DNA and generates a fluorescence signal proportional to the amount of specific PCR product generated. These experiments quantified HCV RNA relative to beta actin expression. This PCR assay was useful in initial experiments, and demonstrated proof-of-principle in the fusion technique. However, quantification using SYBR Green is potentially limited at low copy number by non-specific product, such as primer dimer formation. Quantification of HCV RNA relative to B actin expression does not give a measure of viral replication which is easily comparable to other replication assays. To allow accurate quantification of HCV copy number in further fusion experiments, a sensitive and specific quantitative real time PCR assay was developed.

To enhance sensitivity for detection of low-level viral RNA, a one-step reverse transcription-quantitative PCR (RT-qPCR) assay was employed using a commercial kit dedicated to detection of viral nucleic acids (details in chapter 2). To ensure specificity of PCR product amplification and detection, TaqMan chemistry using a commercial HCV primer and probe set was used (see chapter 2). The addition of the target-specific probe diminishes the occurrence of non-specific product causing a false positive signal, compared to use of SYBR Green reaction chemistry.

Serial dilutions of a known copy number standard in each PCR reaction are required for reliable quantification of HCV copy number. An RNA standard was

therefore synthesised by transcription of JFH-1 RNA from a plasmid containing JFH-1 under a T7 promoter element (see chapter 2). The quantification range for the assay was 10^{-10^8} HCV copies per reaction and the RNA standard amplified with comparable efficiency to samples from fusion experiments, thus was suitable for quantification of HCV RNA in the experimental samples (Fig 3-1 A, B). Estimates of the intra-assay and inter-assay variation of the RT-qPCR assay were generated using standard RNA and expressed as percentage coefficient of variation for copy number (%CV) [279]. Intra-assay variation was calculated from results of PCR triplicates of the RNA standard dilution series in a single PCR experiment. Inter-assay variation was calculated from PCR duplicates of the RNA standard dilution series in 10 independent PCR experiments. The mean intra-assay variation was 25.5%CV and the inter-assay variation was 20.6%CV (Fig. 3-1 C). Intra-assay variation was similar for two separate experimental samples, where over 20 PCR replicates were performed per sample in a single PCR experiment (16.1%CV and 19.0%CV) (Fig 3-1 D). This degree of variation was deemed to be acceptable.

Normalisation of PCR data is performed to control for differences between experimental samples, such as quantity of input RNA or efficiency of reverse transcription. When quantifying expression of cellular mRNA, results are usually normalised to expression of one (or more) reference genes which are stably expressed throughout the experimental conditions under investigation. However, quantification of viral RNA represents a rather different situation as viral RNA production does not necessarily bear any relation to expression of cellular mRNA. In these circumstances, standard practice [280] is to normalise viral RNA to

number of input cells (if known) or total input RNA, measured using a robust method [279]. We chose to express HCV RNA as ‘copies per microgram total RNA’ as quantification of RNA is more accurate than cell counts in this experimental model as this approach avoids the problems associated with cell death in the traumatic fusion process.

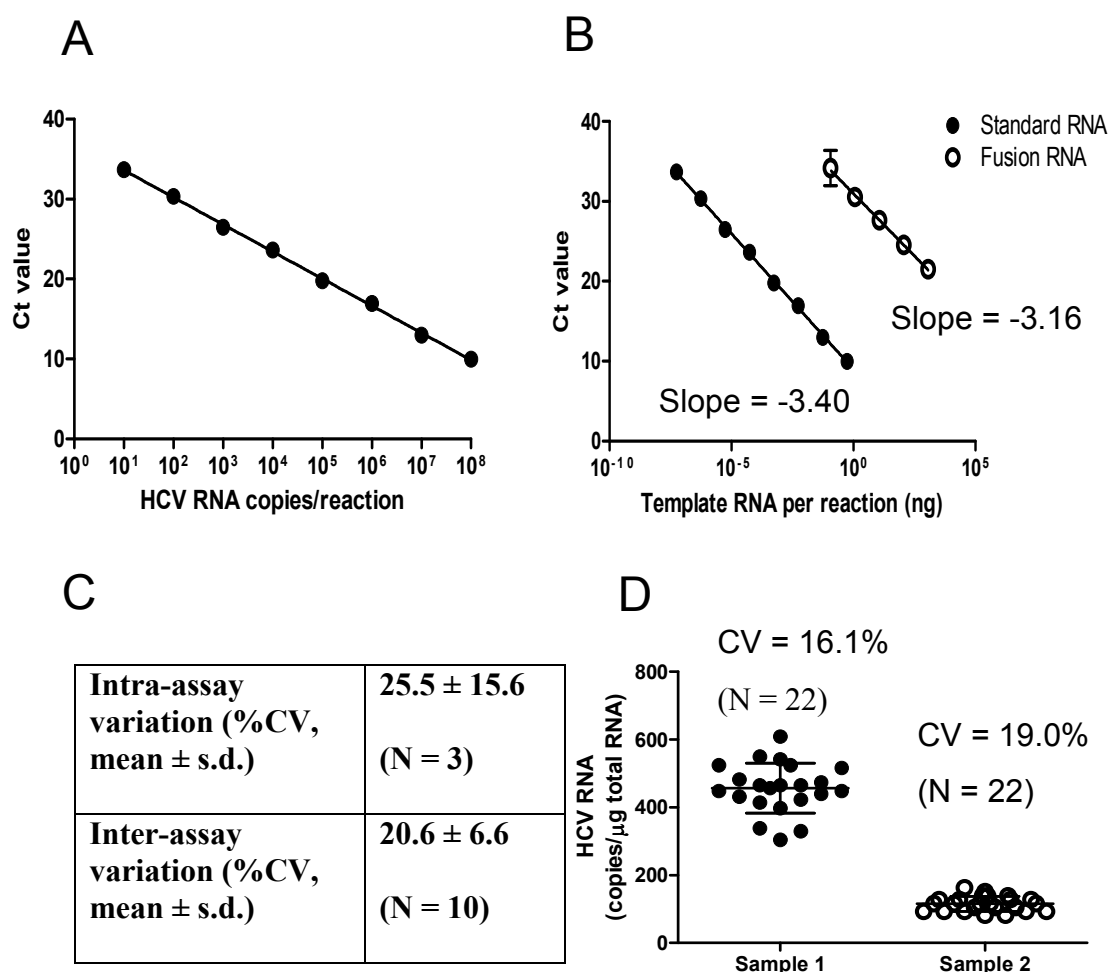


Figure 3-1. Validation of the RT-qPCR assay used for HCV quantification. **A**, standard curve obtained using serial dilutions of a HCV RNA standard. A quantification range of $10 - 10^8$ copies per reaction was obtained. **B**, serial dilutions of RNA from a capture-fusion experiment were run in a PCR reaction alongside serial dilutions of the RNA standard. The difference between slopes was not significant (standard -3.40 ± 0.04 , fusion -3.12 ± 0.18 , $p = 0.072$), indicating comparable reaction efficiency between standard and experimental samples. Graphs **A** and **B** show mean \pm s.d. of PCR triplicates per RNA dilution. **C**, intra-assay and inter-assay variation of the PCR assay, expressed as percentage coefficient of variation (%CV) of the HCV RNA copy number [3]. Intra-assay variability was calculated from PCR triplicates of the RNA standard dilution series shown in **A**. Inter-assay variability was calculated from serial dilutions of RNA standard run in 10 separate PCR reactions. **D**, intra-assay variation was further defined using experimental samples. 22 replicates of two samples of capture-fusion RNA were measured in a single PCR assay. Graph shows mean \pm s.d. with intra-assay variation expressed as %CV for each sample.

3.3 HCV replication can be detected in monocytes from patients infected with diverse viral genotypes

To establish whether the fusion technique could be used to identify viable HCV RNA in monocytes of patients infected with diverse viral genotypes, CD14(+) monocytes were isolated from 8 patients infected with genotype (G)1 HCV and 6 patients with G3 HCV. Details of HCV subtype were not collected at the time of sampling and was not available retrospectively. HCV RNA was quantified at various times after fusion of these monocytes with Huh7.5 cells. An increase in HCV RNA was observed in the majority of samples tested (12 of 14), up to 7 days after fusion, suggesting viral replication. No increase in viral RNA was seen in patient monocytes cultured alone, or in monocytes co-cultured with Huh7.5 cells, without the fusion step, indicating that fusion is required for replication to occur (Fig 3-2). In most experiments the cultured cells reached confluence by day 7 after fusion, most likely due to replication of unfused Huh7.5 cells. Fused cells did not survive passage, and so culture periods longer than 7 days after fusion were not studied.

Prolonged PEG exposure is cytotoxic, and it is possible that fusion-related cytotoxicity could cause an artefactual increase in HCV RNA after fusion due to a relative reduction in cellular RNA. Cell viability experiments performed at various times after fusion confirmed a degree of PEG-induced cytotoxicity, however viability of fused cells increased over the incubation period, mirroring the rise in viral RNA seen. This suggested that PEG-induced cytotoxicity was not a significant contributor to the observed rise in HCV RNA (Figure 3-3).

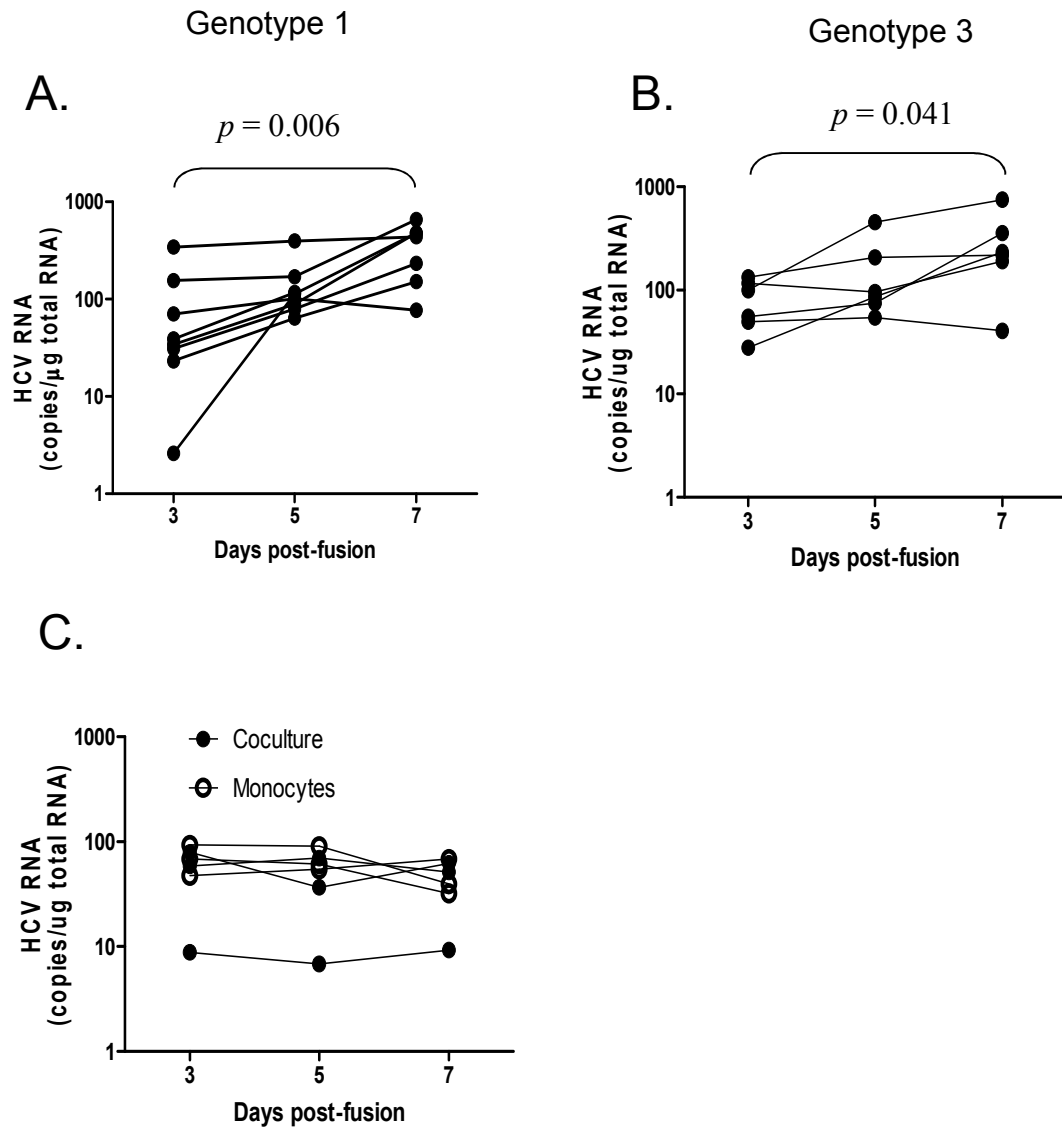


Figure 3-2. HCV replication occurs in patient-derived monocytes fused with Huh7.5 cells. **A, B;** 14 independent experiments demonstrating an increase in HCV RNA copy number after fusion in patients with chronic genotype 1 (subtype unknown) (**A; N = 8**) or genotype 3 (**B; N = 6**) HCV infection. **C,** no evidence of HCV replication was seen when monocytes from 3 of these patients were cultured alone or with Huh7.5 cells, without fusion. p values were calculated using Mann-Whitney U test.

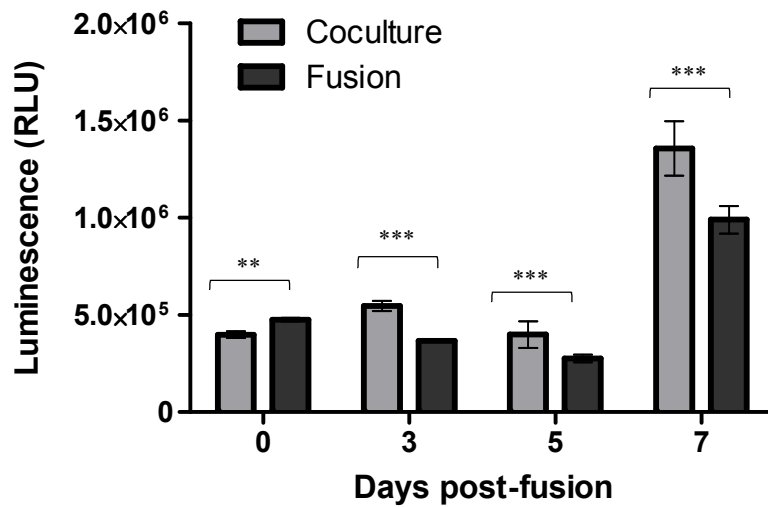


Figure 3-3. Effect of cell fusion on viability of patient-derived monocytes and Huh7.5 cells. An equal number of patient-derived monocytes and Huh7.5 cells were either fused or cultured together without fusion, in biological triplicates. Cell viability was measured after various lengths of time using a luminescent ATP quantification assay. Luminescence was measured in quadruplicate for each sample. Graph shows mean luminescence \pm s.e.m., corrected for background. ** $p = <0.01$; *** $p = <0.0001$. Graph shows results from a single representative experiment. p values calculated using Mann-Whitney U test.

3.4 Alternate strategies to detect HCV replication

An increase in HCV RNA was seen in the majority of patient monocytes after fusion with Huh7.5 cells, suggestive of viral replication. Rather than the logarithmic increases in HCV RNA observed in propagation of cell culture HCV strains, we observed a 2-10 fold increase in viral RNA over 7 days after fusion, suggestive of low level viral replication (Fig 3-2 A,B). Further evidence of HCV replication was sought by using alternate techniques to demonstrate the presence of viral RNA or protein production in fused cells.

3.4.1 Indirect immunofluorescence

To complement the results demonstrating increased HCV RNA in fused cells over time after fusion, fused cells were examined for HCV protein production by

indirect immunofluorescence. Five days after fusion, cells were fixed and stained for the viral NS5A protein and albumin (a Huh7.5 cell marker). Huh7.5 cells infected with the HCV cell culture strain JFH-1, and uninfected Huh7.5 were included as positive and negative controls, respectively. In these experiments, viral NS5A appeared to co-localise in the cytoplasm with albumin, a Huh7.5 cell marker. However, in subsequent experiments including monocytes from uninfected donors fused with Huh7.5 as a negative control, NS5A staining was also identified. We speculated that this might be due to the high affinity of monocytic receptors, particularly FcR, for antibody binding. A number of alternate strategies and protocol variations were employed in an attempt to reduce this non-specific staining, including attempts to detect viral NS3, core and NS5A proteins and a variety of fixing and blocking steps (including use of Fc blockers; experiments performed in conjunction with Dr Meleri Jones, data not shown). Despite these measures, we were unable to eliminate non-specific staining, presumably due to antibody binding to Fc receptors on monocytes within the fused cells, and so were unable to conclusively demonstrate HCV protein production in the fusion model.

3.4.2 Detection of protein production by Western blot

Attempts were made to detect HCV NS3, core and NS5A proteins in fused cells by western blotting. Using a replicon containing cell line, HCV proteins could be detected from cell lysates using this technique (ranging from 10-50 µg total protein), but could not be detected in cell lysates from fused cells (Western blot experiments performed by Dr Meleri Jones, data not shown).

3.4.3 Huh7-J20 reporter cell line

A novel reporter cell line to detect and quantify HCV replication in cell culture has recently been described (Huh7-J20), consisting of Huh7 cells which stably express a fluorescent marker fused to secreted alkaline phosphatase (SEAP) via a sequence which is recognised and cleaved by HCV NS3/4A serine protease. In the presence of HCV replication and free HCV NS3/4 within the cellular cytoplasm, the recognition site is cleaved by NS3/4A and SEAP released into the supernatant, where it can be detected and quantified. The Huh7-J20 reporter cell line has been used to sensitively quantify replication of cell culture HCV (HCVcc) strains [281].

The Huh7-J20 cell line was investigated to see if it could be used as an alternate detection technique for HCV replication in the fusion assay. Huh7-J20 cells were kindly supplied by Dr Arvind Patel (Centre for Virus Research, University of Glasgow). First, a serial dilution of positive control placental alkaline phosphatase was run in the SEAP detection assay to establish its linear range and sensitivity (Fig 3-4A). Huh7-J20 cells were then infected with JFH-1 at 3 separate dilutions, corresponding to concentrations detected by the reporter cell line in published work [281]. After 72 hours, supernatants were harvested for SEAP quantification. In parallel, total RNA was extracted from the infected Huh7-J20 cells for quantification of HCV RNA by RT-qPCR. A dose-dependent increase in SEAP was seen in supernatants from Huh7-J20 cells infected with increasing concentrations of JFH-1, and this mirrored a dose-dependent increase in HCV RNA detected from the cells by RT-qPCR (Fig 3-4 B, C). However, the lowest infectious dose of JFH-1 that was used produced HCV RNA levels approximately

10-fold greater than the highest yields achieved after cell fusion. To determine whether this assay detected replication in the fusion assay, patient monocytes were fused with Huh7-J20 cells. At various times after fusion, supernatants were removed to assay for SEAP and in parallel total RNA was extracted from the cells and HCV RNA quantified by RT-qPCR. Although an increase in HCV RNA was seen over time after fusion by RT-qPCR, no change above background was seen using the SEAP assay (data not shown). Thus whilst the Huh7-J20 reporter cell line could detect and quantify replication of ccHCV, it was not suitable for use as a tool to detect HCV replication after fusion of patient monocytes with Huh7.5 cells.

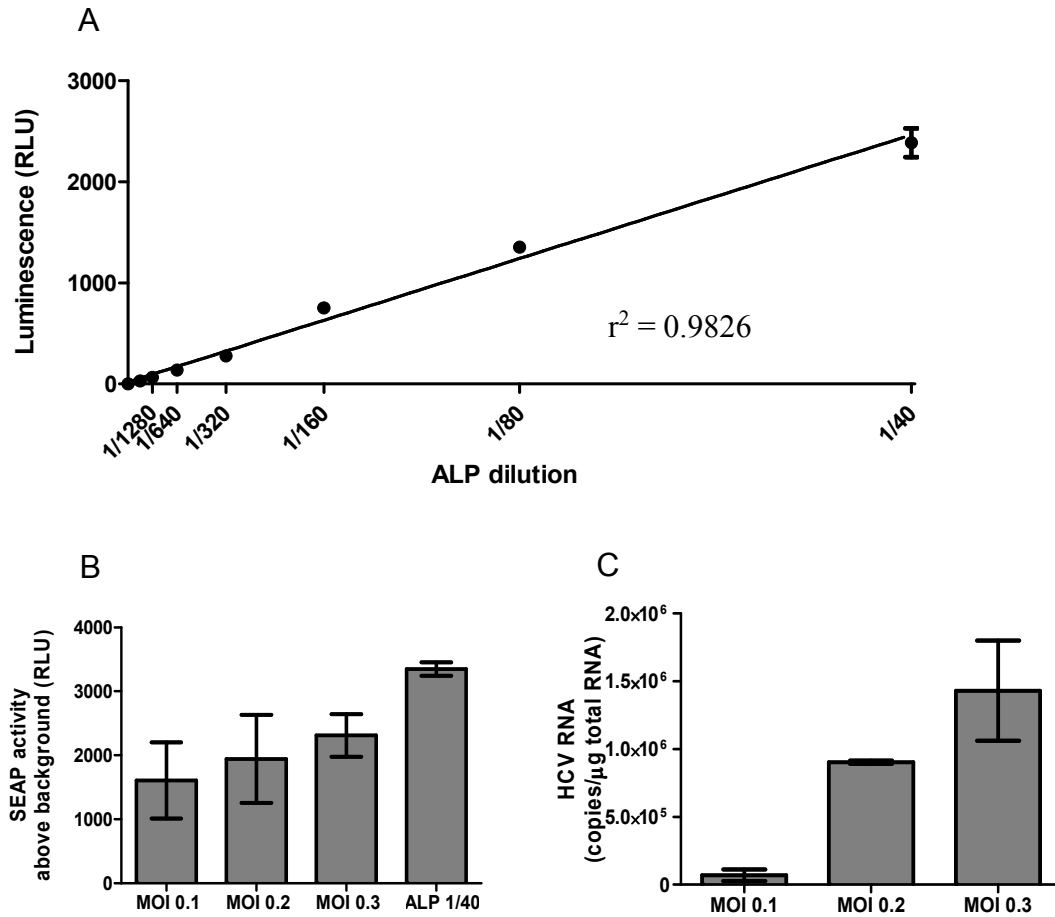


Figure 3-4. Detection of JFH-1 replication by the Huh7-J20 cell line. **A.** Serial dilutions of placental alkaline phosphatase (ALP) positive control confirmed that luminescence was proportional to alkaline phosphatase over a wide linear range. **B.** SEAP activity detected as relative light units (RLU) 72 hours after infection of Huh7-J20 cells with JFH-1 at a range of infectious doses. ALP was included as a positive control at a dilution of 1/40. **C** shows the corresponding HCV RNA yield measured by RT-qPCR from Huh7-J20 cells infected with various doses of JFH-1 for 72 hours, after the supernatants had been removed to assay for SEAP. Graphs show mean \pm sd of biological triplicates. Graphs each show results from a single representative experiment. R^2 calculated using linear regression analysis.

3.5 Enhancement of HCV replication by isolation of monocyte subsets for fusion

Although a consistent increase in HCV RNA was seen after fusion of monocytes from most patients with chronic HCV, the level of replication remained relatively low and close to the quantification limit of the PCR assay. Human monocyte subpopulations can be categorised according to CD14 and CD16 expression [282]. Phenotype and function of these subpopulations is poorly characterised,

however one report has suggested that HCV infects both CD14+CD16++ and CD14++CD16++ cells, but not CD14+CD16- cells [271]. If so, isolation of the CD14+CD16+ subpopulation for fusion might enhance viral replication by enriching the population of infected monocytes for fusion. Total PBMCs from a patient with chronic HCV infection were labelled with fluorescence-conjugated anti-CD14 and anti-CD16 monoclonal antibodies. CD14+CD16- and CD14+CD16+ monocyte subsets were isolated by fluorescence activated cell sorting (FACS). Each cell population was fused with Huh7.5 cells and HCV RNA compared after 3 and 5 days. Due to the small number of cells in each population, further time points after fusion were not analysed. There was no significant difference in HCV RNA obtained 3 or 5 days after fusion of the CD14+CD16+ cells compared to CD14+CD16- cells (Fig. 3-5). Given that isolation of monocyte subpopulations for fusion did not significantly enhance HCV RNA replication, all further fusion experiments were performed using total monocytes.

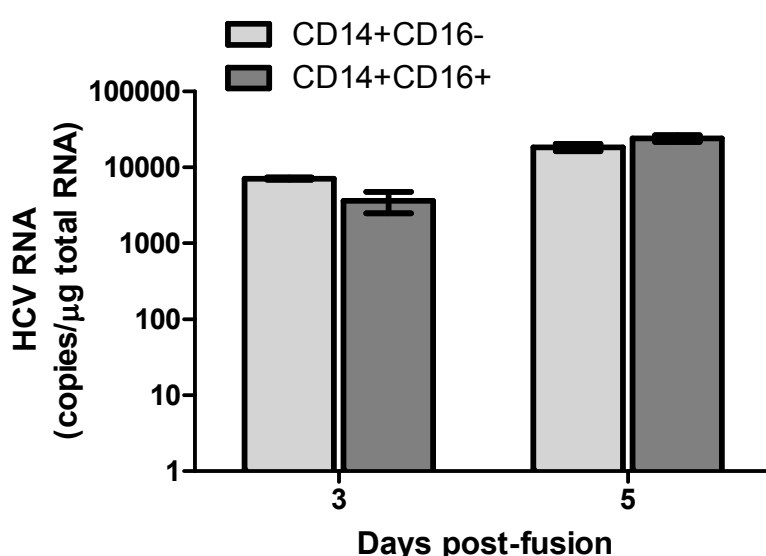


Figure 3-5. Separation of monocyte subsets did not enhance HCV replication after fusion. CD14+CD16+ or CD14+CD16- monocytes were isolated from total PBMCs from a patient with chronic HCV infection by FACS. Each subpopulation was fused with Huh7.5 cells and HCV RNA quantified 3 and 5 days after fusion. Graph shows mean \pm sd of biological triplicates from a single experiment.

3.6 HCV replication in fused cells is inhibited by antiviral drugs

Inhibition of viral replication with a reduction of HCV RNA provides valuable confirmation of viral replication. Monocytes isolated from patients with chronic G1 HCV infection were fused with Huh7.5 cells and then cultured in the presence of interferon α -2a (IFN) or the HCV protease inhibitor telaprevir. HCV RNA was quantified and compared with untreated cells at various time points after fusion. By day 7 after fusion, both antiviral drugs significantly reduced detectable HCV RNA compared to untreated cells, suggesting inhibition of viral replication (Figure 3-6).

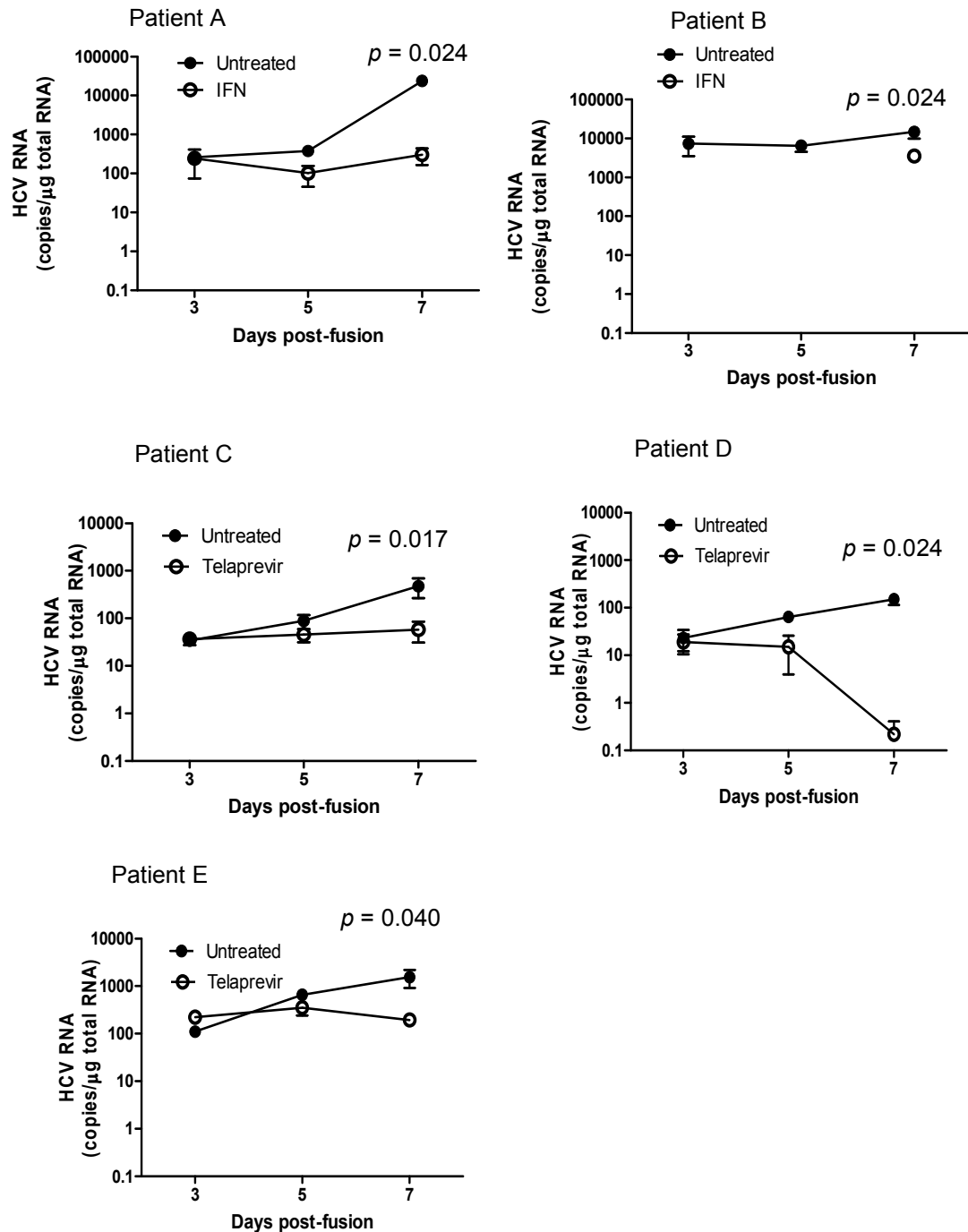


Figure 3-6. Inhibition of HCV replication in fused patient monocytes with antiviral drugs. Monocytes from 5 patients with chronic G1 HCV were fused with Huh7.5 cells, and cultured in the presence or absence of either interferon-2a (IFN; patients **A** and **B**) or telaprevir (patients **C**, **D** and **E**) for up to 7 days. By 7 days after fusion significantly less HCV RNA was obtained from drug treated than untreated cells from all patients. Due to a low yield of monocytes, only day 7 data for IFN treated fused cells was available for patient B. Graphs show mean \pm s.e.m of single independent experiments. All p values are for comparisons of drug treated and untreated cells at day 7 after fusion and were calculated using Mann-Whitney U test.

3.7 Viable HCV in patient monocytes at the end of treatment as a predictor of relapse

Previous work in our group has demonstrated that when patients treated with pegIFN/ribavirin for chronic HCV infection relapse, this occurs rapidly after the end of treatment. Detectable viraemia is seen within a week of treatment cessation and viral load returns to pre-treatment levels within 4-6 weeks (Dania Shoeb, unpublished data). This is compatible with a model in which HCV persists in a sanctuary site during treatment, with rapid emergence and re-establishment of infection after withdrawal of therapy. Monocytes have been implicated as a sanctuary site for viral relapse in patients co-infected with HIV and HCV [146]. Given our findings that monocytes harbour viable HCV during chronic HCV mono-infection, we hypothesised that monocytes may also act as a sanctuary site during treatment of mono-infected patients and may be associated with or responsible for relapse. This section describes experiments performed to test the hypothesis that the presence of viable HCV in patient monocytes at the end of therapy, detected using the fusion technique, predicts relapse in patients treated for chronic HCV infection.

3.7.1 Predicting relapse in G3 HCV after treatment with pegylated interferon and ribavirin

Patients with chronic G3 HCV infection who fail pegIFN/ribavirin treatment most often respond to therapy initially, but then relapse once treatment is withdrawn. This is in contrast to G1 HCV infection, where the more common pattern of treatment failure is primary non-response or partial response to pegIFN and

ribavirin (discussed in more detail in Chapter 1). Due to the higher rate of treatment failure due to relapse, patients with G3 HCV were selected for these experiments.

PBMCs were taken from 18 sequential patients attending their end of treatment visit. All patients had completed a standard course of 24 weeks pegIFN/ribavirin treatment for chronic G3 HCV. CD14⁺ monocytes were isolated and fused with Huh7.5 cells. HCV RNA was quantified in unfused monocytes and at 3, 5 and 7 days after fusion, in duplicate or triplicate, depending on the number of monocytes obtained. Treatment outcome information was obtained for each patient 6 months after the end of treatment, either relapse or sustained virological response (SVR).

Patient demographics are shown in Table 3-1, according to treatment outcome. Participants were predominantly female in both groups. Relapsers were older with slightly greater fibrosis scores than patients with SVR. Two of the patients who relapsed (33%) had biopsy-confirmed cirrhosis whilst none of the patients with SVR were cirrhotic. Pre-treatment viraemia did not differ between the groups.

	SVR (N = 12)	Relapse (N = 6)	<i>p</i> value
Female:Male	8:4	4:2	
Age (years)	41.5 (28 – 50)	54.5 (41 – 63)	0.019
Pre-treatment viral load (IU/mL)	4.16x10 ⁵ (6.95x10 ⁴ – 1.45x10 ⁷)	2.23x10 ⁵ (1.18x10 ⁴ – 6.93x10 ⁶)	0.815
Fibrosis score	1 (0 – 3)	3.5 (1 – 6)	0.041

Table 3-1. Baseline characteristics of patients submitting end-of-treatment monocytes, according to ultimate treatment outcome. Fibrosis score was expressed using the Ishak scoring system. Values are given as median (range). SVR, sustained virological response. *P* values were calculated using Student's t-test.

HCV RNA obtained from fused cells was compared between patients with SVR and those with relapse. Results were compared at baseline (unfused monocytes) and 5 days after fusion to determine whether or not viral replication (as evidenced by an increase in HCV RNA) occurred. The change in HCV RNA from day 0 to day 5 after fusion was significantly greater in patients who relapsed than patients with SVR (expressed as % change to normalise for variability in baseline HCV RNA between samples; Fig 3-7A). HCV RNA increased from day 0 to day 5 after fusion in fused monocytes from patients who relapsed, although this did not reach statistical significance in this small number of samples. Similarly, HCV RNA decreased after fusion in fused monocytes from patients who achieved SVR, although again this did not reach statistical significance (Fig 3-7B, C).

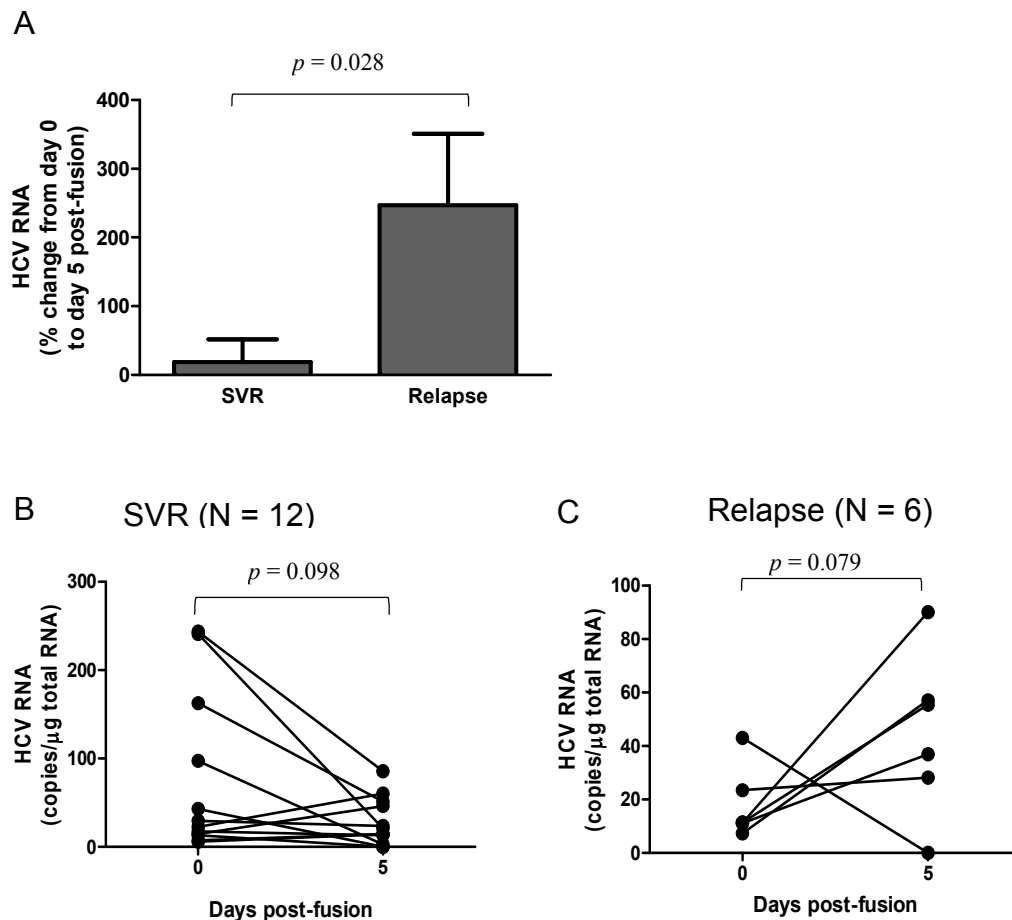


Figure 3-7. Fusion of end of treatment monocytes according to treatment outcome. End-of-treatment monocytes from patients with G3 HCV treated with pegIFN/ribavirin were fused with Huh7.5 cells and HCV RNA quantified at Day 0 and Day 5 post-fusion. The percentage change in HCV RNA from Day 0 to Day 5 post-fusion was significantly greater amongst fused monocytes from relapsers than from patients with SVR (**A**). HCV RNA declined over time after fusion of monocytes from patients who achieved SVR (**B**; N = 12), and increased after fusion of monocytes from patients who relapsed (**C**; N = 6), although these changes did not reach statistical significance in this small cohort of patients. *P* values were calculated using Mann Whitney U test.

3.7.2 Predicting relapse in G1 HCV after treatment with sofosbuvir and ribavirin

Sofosbuvir is a nucleotide analogue inhibitor of HCV RNA-dependent RNA polymerase which has shown potent anti-HCV activity in clinical trials and has recently been licensed for treatment of chronic HCV (discussed in more detail in

chapter 1). Clinical trials of sofosbuvir in chronic G1 HCV have shown high efficacy when this drug is combined with pegIFN and ribavirin [205, 206, 238], but a significant rate of relapse when given with ribavirin alone [235].

To investigate whether monocytes could act as a sanctuary site for HCV during sofosbuvir and ribavirin treatment, stored, end of treatment PBMCs were kindly supplied by Dr Shyam Kottlil (NIH, Bethesda) from 8 patients with G1 HCV who had received sofosbuvir/ribavirin in a clinical trial, with differing treatment outcomes (4 patients who achieved SVR and 4 who relapsed). CD14⁺ monocytes were isolated and fused with Huh7.5 cells. As these patients had not received interferon, the monocyte yield was generally high and sufficient cells were obtained to measure HCV RNA up to 7 days after fusion. HCV RNA yield over time after fusion was compared between patients with SVR and those who relapsed. Regardless of treatment outcome the yield of HCV RNA decreased over time after fusion, with no significant difference between samples from patients with SVR and relapse after sofosbuvir/ribavirin therapy (Fig 3-8). This suggests absence of viable HCV associated with monocytes at the end of treatment, and that HCV sequestration in monocytes during sofosbuvir/ribavirin therapy is not a mechanism for relapse in these patients.

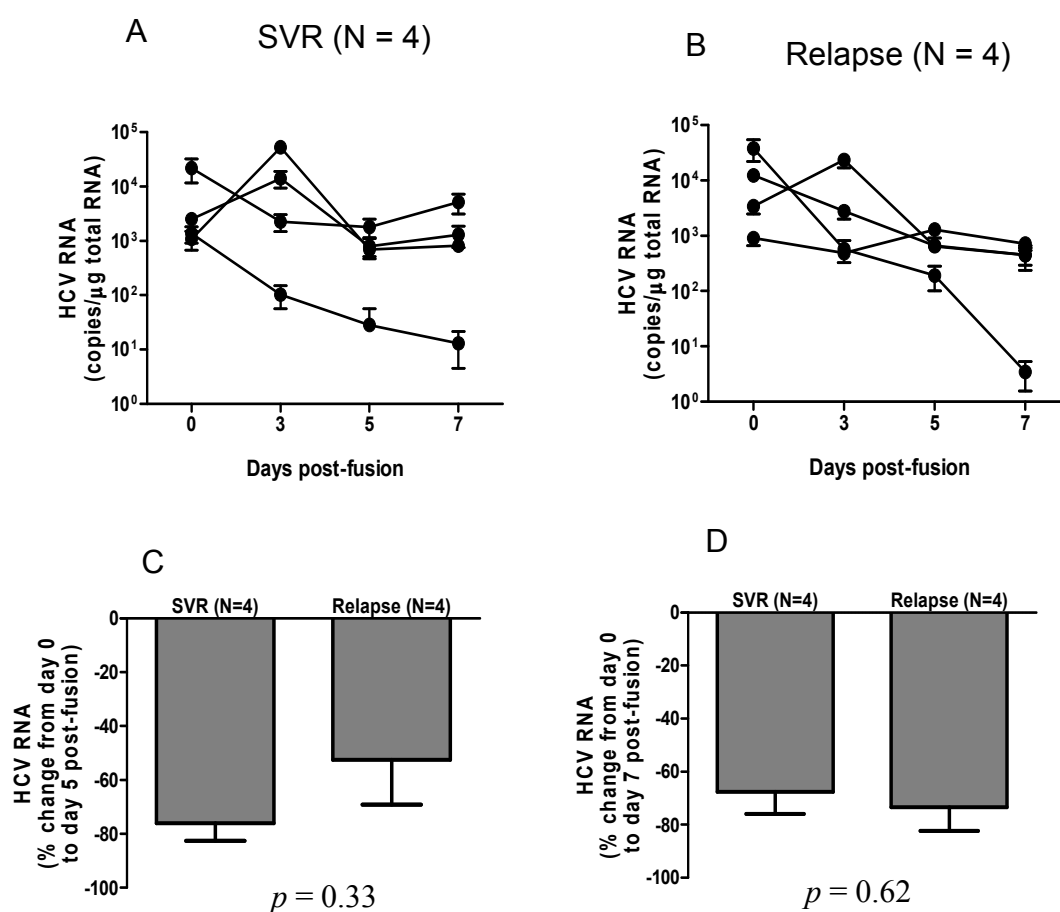


Figure 3-8. Absence of viable HCV in monocytes at the end of treatment in patients with G1 HCV treated with sofosbuvir and ribavirin. CD14⁺ monocytes were isolated from end of treatment PBMCs from 4 patients with SVR and 4 patients who relapsed after sofosbuvir and ribavirin treatment in a clinical trial. After fusion with Huh7.5 cells, HCV RNA was quantified at days 0, 3, 5 and 7 post-fusion and compared between patients with SVR (**A**) and relapse (**B**). There was no significant difference in the change in HCV RNA from day 0 to day 5 (**C**) or day 0 to day 7 after fusion (**D**) between patients with different treatment outcomes. Graphs each show mean \pm s.e.m. of 4 independent experiments. *P* values were calculated using Mann Whitney U test.

3.8 Discussion

This chapter builds on previous work from our group, in which a technique for fusion of patient-derived monocytes with Huh7.5 cells was developed and optimised as a means to detect HCV replication. Development of a highly sensitive and specific quantitative real time PCR technique allowed quantification of low level HCV RNA copy number. This was used to document an increase in HCV RNA over time after fusion, as well as inhibition of this increase by antiviral drugs, both of which are suggestive of HCV replication after fusion.

Technical difficulties in detection of HCV RNA at low copy number have long been recognised as a challenge to investigation of extrahepatic HCV replication using other techniques, such as negative strand PCR or core antigen assay [283]. These techniques were not attempted in view of technical difficulty and poor sensitivity (for example, the sensitivity of negative strand PCR has been reported by various authors to be 10-1000 fold less than that of the positive strand assay; reviewed in [108]), as even after cell fusion the level of HCV replication remained low. Instead, presence of replicating virus has been strongly suggested by increase in viral RNA over time which was inhibited by treatment of the fused cells with antiviral drugs. Together, these data suggest that the fusion technique complements existing literature on detection of HCV in extrahepatic sites and support the presence of replication competent HCV RNA associated with monocytes from patients with chronic HCV infection.

Attempts to use alternate techniques to demonstrate and quantify HCV replication after fusion (Western blotting, indirect immunofluorescence and the

Huh7-J20 reporter cell line) were unsuccessful. The most likely explanation is that these techniques were insufficiently sensitive to detect HCV RNA in the fusion model. Using Western blotting, HCV proteins could be detected in extracts from replicon cells, which support high level viral replication, but the signal was lost when the cell extracts were diluted to a level approximating the HCV yield seen in the fusion experiments. Detection of HCV proteins by indirect immunofluorescence in fused cells was complicated by persistent non-specific background staining, attributed to high avidity of monocytes for antibody binding. The Huh7-J20 cell line has been noted to be less permissive for HCV replication than the parent cell line [281], which may have a significant impact on comparatively low-level replication seen after fusion. Alternatively, as this cell line has been reported to be as sensitive as RT-qPCR for detection of HCVcc [281], it is possible that virus transferred to Huh7-J20 cells by cell fusion is masked from the SEAP reporter construct, for example in an alternate intracellular compartment. At present, the low level of HCV replication after fusion has precluded investigation of its subcellular localisation but this represents an area for further study.

Attempts were made to enhance HCV replication after fusion, in particular by isolating the CD14+CD16+ monocyte subpopulation which has been suggested to be preferentially infected with HCV [271]. Following fusion, no difference was seen in the yield of HCV RNA from CD14+CD16+ or CD14+CD16- monocytes. These discordant findings could be explained by differences in the detection systems used. The paper reporting absence of positive strand HCV RNA in CD14+CD16- monocytes used a sensitive in situ hybridisation technique to detect

HCV RNA in patient monocytes [271], but it is difficult to establish how sensitive this assay was in comparison to the RT-qPCR assay used in the fusion experiments. It is possible that viable HCV RNA present at a very low level in CD14+CD16- monocytes may not have been detected by this in situ hybridisation technique. Even if present at lower levels initially, following fusion with Huh7.5 cells, replication of viable HCV present in this subpopulation may have “caught up” with that of virus in CD14+CD16+ cells, especially if other blocks to viral replication in fused cells created a ceiling for replication. The very small number of monocytes obtained in each subpopulation after cell sorting meant that no unfused monocyte RNA was available for HCV RNA quantification to explore this possibility further.

PEG has been used to induce cell fusion for over 3 decades [278] and is established as a method of combining cell characteristics in generation of hybridomas. The method by which PEG induces cell membranes to fuse is incompletely understood, but is thought to involve exclusion of water molecules from membranes at areas of close contact [278]. Cell fusion techniques have been employed by others to investigate extrahepatic HCV replication [284]. However, cell fusion has not previously been used to facilitate replication of patient-derived HCV. We found that transfer of HCV associated with patient monocytes to Huh7.5 cells by cell fusion facilitated replication. Whether this was due to removal from an environment rich in restriction factors, or transfer to an environment containing permissive factors, or both, has not been established. The presence of dominant restriction factors has been excluded as the primary block on HCV replication in certain non-permissive human or murine cells [284],

however data on monocytes is lacking. The Huh7 hepatoma cell line is uniquely permissive for HCV replication, especially Huh7.5 cells which were generated by curing a highly permissive Huh7 subclone [276]. The factors underlying the permissiveness of Huh7.5 to HCV replication are not fully understood, but are thought to include defective innate anti-viral signalling (in particular RIG-I signalling) [285] and presence of the liver-specific microRNA miR-122, which appears critical for efficient HCV replication [55].

The observation that HCV replication occurs after fusion of patient monocytes with Huh7.5 cells implies that monocytes of patients with chronic HCV infection harbour viable HCV. Whether this monocyte-sequestered virus may be responsible for relapse after cessation of antiviral therapy had not previously been examined in HCV mono-infected patients. Relapse was the predominant mechanism of treatment failure amongst patients with G3 HCV treated with pegIFN/RBV and also amongst patients with G1 HCV treated with sofosbuvir/RBV. The presence of viable HCV in end-of-treatment monocytes appeared to be associated with relapse amongst G3 patients treated with IFN/RBV, but not G1 patients treated with sofosbuvir and ribavirin. Whether this discrepancy is due to genotypic differences or to the treatment regimen would require further study of patients with G1 HCV treated with pegIFN/RBV, and patients with G3 infection treated with sofosbuvir/RBV. This may be difficult to achieve in practice as the standard of care for G1 patients moves away from pegIFN/RBV alone to regimens containing new antiviral agents, and as interferon is recommended together with sofosbuvir/RBV for patients with G3 infection [155]. It is interesting to note that although no indication of viable HCV RNA was

found in monocytes from patients treated with sofosbuvir and ribavirin, the amount of HCV RNA detected by the PCR assay was considerably higher than that detected from G3 patients treated with pegIFN/RBV. The reasons for this could be multifactorial, relating to storage of samples, some differences in reagents used (G1 experiments were performed at the NIH laboratories in Bethesda, although the extracted RNA was shipped to London for HCV quantification). Genotypic differences are also possible, although less likely as this was not observed amongst patients infected with chronic HCV of different genotypes. It is possible that IFN treatment may have enhanced uptake and degradation of non-viable HCV virions or RNA fragments by monocytes and other immune cells and following IFN-free therapy, this viral detritus remains detectable. Although these results suggest that monocytes may act as a sanctuary site in G3 patients treated with IFN/ribavirin, the lack of viable HCV associated with monocytes of patients who relapsed after sofosbuvir/RBV therapy suggests that other mechanisms are involved in relapse after therapy and other sanctuary sites may be as, or more, important. Alternatively, it remains possible that sofosbuvir-resistant HCV does persist in monocytes of patients who relapsed at the end of therapy, but this virus is poorly replication-competent and so could not be detected in the fusion model, which supports low-level viral replication at best.

Sequencing of sequestered virus in monocytes and comparison with viral sequences present in serum before treatment/after relapse would provide further insights into the role of HCV RNA detectable in end of treatment monocytes in relapse. Unfortunately, extensive attempts to sequence HCV RNA from

monocytes of G3 patients at the end of treatment and after fusion unsuccessful, most likely due to the relatively low yield of HCV RNA obtained (experiments performed by Dr Jenny Waters). With the development of novel sequencing technologies, it should be possible to investigate this further in the near future.

4.0 Results: Development of a “capture-fusion” model to study replication of patient-derived HCV

4.1 Introduction

Our observation that patient monocyte-associated HCV could be induced to replicate in culture, and inhibited with antiviral drugs, led us to question whether this model could be used to assess patient-specific responses to novel antiviral therapies. However there are several practical limitations to the use of patient-derived monocytes for this purpose. For example, a relatively large volume of blood is required from each patient, the yield of cells is relatively low, and sample storage and opportunities to share samples between laboratories are limited. For these reasons we sought an approach which would enable use of patient serum instead of monocytes. This chapter describes development of a “capture-fusion” assay, in which virus from patient serum is captured by a monocytic cell line before fusion with Huh7.5 cells and quantification of HCV RNA.

4.2 Investigation of candidate monocytic cell lines

HCV infection or association with a number of monocytic cell lines has been reported [123, 286, 287]. The monocytic cell lines THP-1 and Monomac 1 were selected for initial evaluation for their ability to capture HCV from patient serum.

4.2.1 Comparison of HCV capture by THP-1 and Monomac-1 cells

THP-1 is a human monocytic cell line derived from peripheral blood of a 1 year old male infant with acute monocytic leukaemia [265] that has been used to study replication of another Flavivirus, Dengue virus, *in vitro* [288]. MonoMac 1 is a human monocytic cell line derived from the peripheral blood of a patient with

acute peripheral monoblastic leukaemia [289], which has been used as a model system for HIV replication [290]. It has previously been reported that uptake of HCV immune complexes into another monocyte cell line (U937) was enhanced by pre-exposure of the cells to a combination of PMA and IFN γ [123]. In initial experiments, THP-1 and MonoMac-1 cells were pre-stimulated with PMA and IFN γ . Empirically determined concentrations of PMA/IFN γ were used, based on published work [123]. Cells were cultured in the presence or absence of PMA/IFN γ for 24 hours before thorough washing. After 24 hours of exposure to PMA/IFN γ , THP-1 cells became adherent to the bottom of the well and no further proliferation was seen. In contrast, MonoMac 1 cells remained in suspension but aggregated in large clusters. After removal of stimulants, the cells were exposed to patient serum for 24 hours, then washed again and HCV RNA measured at various times. Viral association with both THP-1 and MonoMac 1 cells was significantly enhanced by stimulation (Fig. 4-1). However, HCV RNA detectable by RT-qPCR declined over time after exposure, implying absence of viral replication in these cells regardless of PMA/IFN γ stimulation (Fig 4-1). As the MonoMacs aggregated after PMA/IFN γ stimulation, this made them unsuitable for downstream fusion experiments. Further experiments were therefore performed with THP-1 cells only.

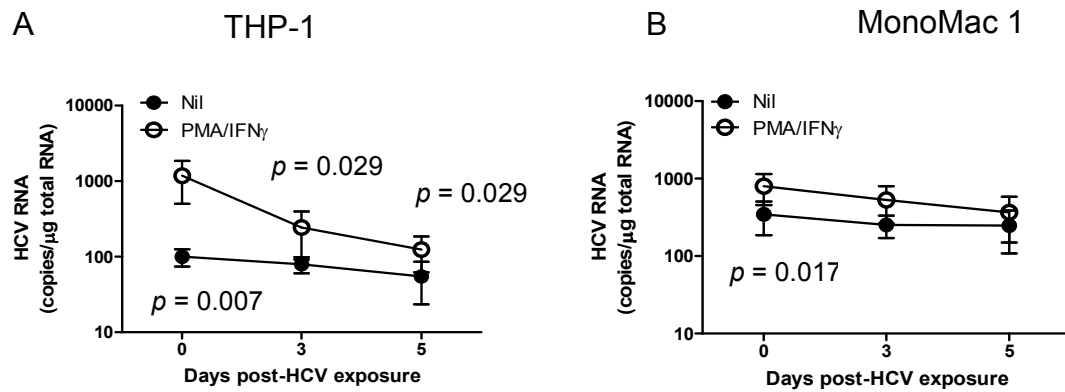


Figure 4-1. HCV does not replicate in monocytic cell lines, but viral association is enhanced by pre-stimulation with PMA/IFN γ . THP-1 (A) or MonoMac1 cells (B) were cultured with PMA (200ng/mL)/IFN γ (10ng/mL) or in the absence of stimulants for 24 hours, then incubated with HCV (+) patient serum for 24 hours. HCV RNA was quantified from the cells up to 5 days after HCV exposure. Graphs show mean \pm s.d. of single representative experiments. *P* values were calculated using Mann Whitney U test.

4.2.2 IFN γ enhances HCV association with THP-1

IFN γ is a cytokine component of the innate antiviral immune response, and would be expected to prime intracellular antiviral responses and potentially inhibit subsequent HCV infection of monocytes. However, IFN γ has also been reported to potentiate uptake of HCV immune complexes into monocytic cells [123]. HCV uptake/association with THP-1 was compared after no stimulation, PMA alone, IFN γ alone or PMA with IFN γ . The combination of PMA and IFN γ led to greatest association of HCV with the cells (Fig. 4-2). Given these findings, stimulation with both PMA and IFN γ was used in all further experiments.

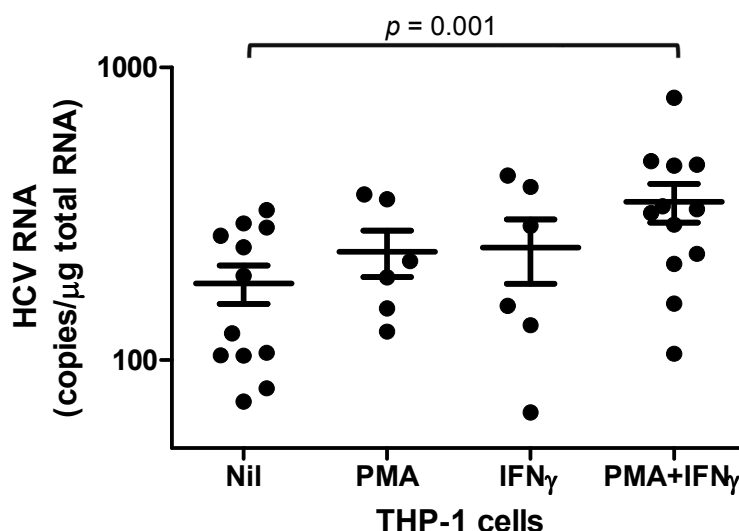


Figure 4-2. Association of patient-derived HCV with THP-1 was enhanced by pre-stimulation with PMA and IFN_γ. THP-1 cells were incubated with PMA (200ng/mL), IFN_γ (10ng/mL) PMA (200ng/mL) and IFN_γ (10ng/mL) or no stimulants for 24 hours, prior to incubation with HCV (+) patient serum for 24 hours then quantification of HCV RNA. Graph shows mean \pm sem of results from 3 independent experiments. *P* value was calculated using Student's t-test.

4.2.3 Patient-derived HCV is internalised into THP-1 cells

Pre-treatment of THP-1 with PMA/IFN_γ enhanced HCV RNA association after exposure to patient serum. To investigate whether the HCV RNA was internalised into THP-1, PMA/IFN_γ-stimulated THP-1 cells were treated with trypsin at various times after exposure to patient serum (experiments performed in conjunction with laboratory technician Joseph Davidson-Wright). Viral internalisation occurred between 1 and 4 hours after exposure to serum, as by 4 hours a proportion of viral RNA could not be removed by trypsin. The proportion of internalised virus did not increase significantly between 4 and 24 hours of infection, indicating that uptake was close to maximal by 4 hours after exposure (Fig. 4-3). These experiments suggested that a minority of HCV RNA was internalised (approximately 20% at 24 hours after exposure), whilst the majority remained at the cell surface.

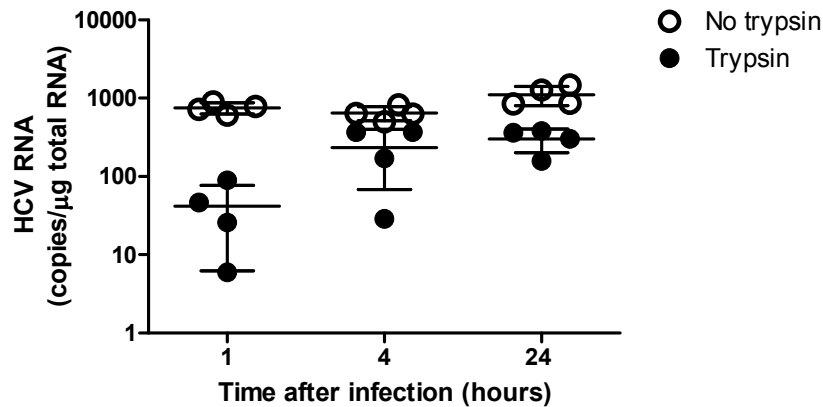


Figure 4-3. Internalisation of HCV RNA by pre-stimulated THP-1 cells. THP-1 cells were incubated with PMA (200ng/mL) and IFN γ (10ng/mL) for 24 hours, before exposure to HCV (+) patient serum. Cells were treated with trypsin at 1, 4 or 24 hours after infection and HCV RNA quantified immediately after. $p = 0.029$ for the comparison between trypsin treated cells at 1 hour and 24 hours after infection. Graph shows mean \pm s.d. of a single representative experiment. P value was calculated using Mann Whitney U test.

4.3 Replication of patient-derived HCV associated with THP-1 after fusion with Huh7.5 cells

Analogous to the experiments on fused patient monocytes (chapter 3), we hypothesised that patient-derived HCV associated with THP-1 cells was viable and could be induced to replicate by transfer to a more permissive environment, by fusion with Huh7.5 cells. Again, we sought evidence of HCV replication by viral RNA accumulation after fusion, inhibition with antiviral drugs, and demonstration of HCV protein production.

4.3.1 HCV RNA accumulation after fusion suggests viral replication

To investigate whether patient-derived HCV RNA associated with THP-1 would replicate after fusion with Huh7.5 cells, THP-1 were stimulated with PMA/IFN γ for 24 hours before thorough washing and incubation with patient serum for 24 hours.

After further washing, these cells were fused with Huh7.5. The hybrid cells were maintained in culture for up to 5 days before quantification of HCV RNA. As controls, Huh7.5 cells and PMA/IFN γ stimulated THP-1 cells were independently incubated with serum from the same patient for 24 hours, then washed thoroughly and maintained in culture in parallel with the fused cells. An increase in HCV RNA was seen over time after fusion, which was not observed in HCV-exposed unfused THP-1 or Huh7.5 cells (Fig. 4-4), suggesting that replication of HCV RNA occurred after capture from patient serum by THP-1 cells and fusion with Huh7.5.

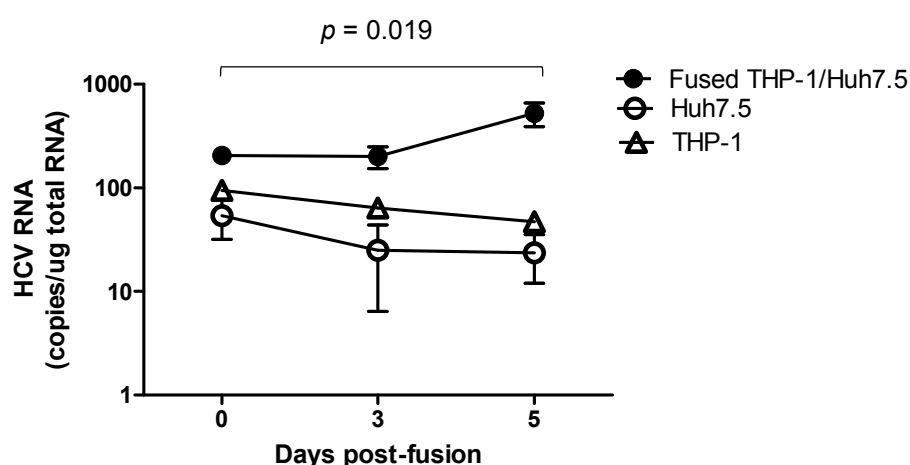


Figure 4-4. Replication of patient-derived HCV after fusion of HCV-exposed THP-1 with Huh7.5 cells. THP-1 were pre-stimulated with PMA 200ng/mL/IFN γ 10ng/mL before exposure to patient serum for 24 hours then fusion with Huh7.5. In parallel, stimulated THP-1 and Huh7.5 cells were exposed to patient serum and maintained in culture without fusion. HCV RNA was quantified from the cells up to 5 days after fusion. Graph shows mean \pm s.e.m of a results from a single representative experiment. $p = 0.019$ for the comparison between fused THP-1/Huh7.5 at day 0 and day 5 after fusion, calculated using Mann Whitney U test.

4.3.2 Antiviral drugs inhibit HCV replication after fusion

Although an increase in HCV RNA was observed after fusion of pre-stimulated, HCV-exposed THP-1 with Huh7.5, the rise was modest. To provide further evidence that this rise reflected low-level replication of patient-derived HCV, the hybrid cells were treated with antiviral drugs after fusion. Initial experiments compared HCV RNA 5 days after fusion where the hybrid cells had been cultured in the presence or absence of interferon α -2a (IFN), or the cyclophilin inhibitor alisporivir. HCV RNA yield 5 days after fusion was significantly reduced by IFN or alisporivir treatment (Fig. 4-5), suggesting inhibition of viral replication by antiviral drugs in the fused cells.

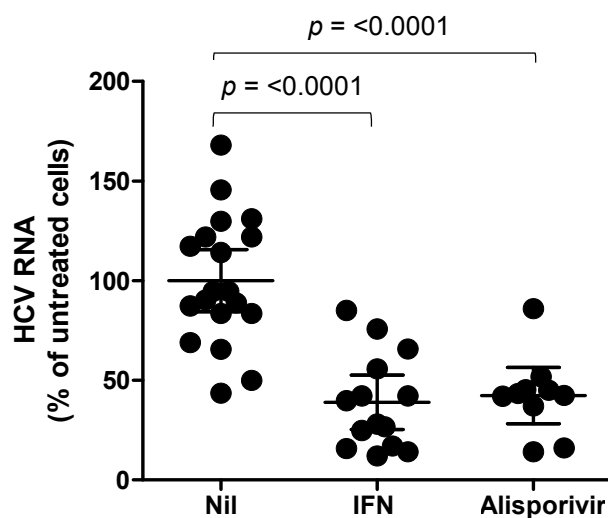


Figure 4-5. HCV yield 5 days after fusion of HCV-exposed THP-1 with Huh7.5 is reduced by treatment with antiviral drugs. Fused cells were treated with IFN 100IU/mL, alisporivir 1.0 μ M, or no drugs for 5 days after fusion prior to quantification of HCV RNA. Graph shows mean \pm 95% CI of results from 2 independent experiments, expressed as percentage of untreated cells to normalise for differences in RNA yield between samples. *P* values were calculated using Student's t-test.

4.3.3 Indirect immunofluorescence

To provide further evidence to support HCV replication after fusion using a technique other than PCR, fused cells were examined for HCV protein production by indirect immunofluorescence. Hybrid cells were fixed and stained for the viral NS5A protein and albumin (a Huh7.5 cell marker) 5 days after fusion. Huh7.5 cells infected with the HCV cell culture strain JFH-1 and uninfected Huh7.5 were included as positive and negative controls, respectively. In these experiments, viral NS5A appeared to co-localise in the cytoplasm with albumin, a Huh7.5 cell marker, suggesting viral replication in fused cells. However, similar to experiments using patient-derived monocytes in cell fusion (see chapter 3), a high level of non-specific background staining was seen when uninfected THP-1 fused with Huh7.5 were used as a negative control. Again, this was thought to be due to the high affinity of monocytic receptors, particularly FcR, for antibody binding. As the level of HCV replication detectable by RT-qPCR was higher in experiments using patient-derived monocytes in fusion than THP-1, and strategies to eliminate background staining had been unsuccessful in those experiments, further attempts to eliminate background staining in THP-1 fusion experiments were not made.

4.3.4 Exclusion of contamination by laboratory HCV strains by sequencing

To exclude contamination by laboratory strains, particularly the high-replicating G2 strain JFH-1, a 265-base region of the HCV 5'UTR from capture-fusion experimental RNA was cloned and sequenced. Details of the methods used are given in Section 2.6.8. Briefly, cDNA derived from RNA samples from 2 separate capture-fusion experiments using G1a donor serum was amplified by PCR using

a high-fidelity reverse transcriptase and PCR primers designed specific to the reference G1a strain H77. Small aliquots of the PCR product were run on a 1% agarose gel. Although no bands were seen in the experimental samples, a band was present in the positive control reaction and the lack of visible band in the experimental samples was thought to be likely to be due to low level of PCR product present. A second round of PCR was not performed due to concerns regarding introduction of contamination. After purification and concentration of the PCR reaction product, this was introduced into TOPO vectors and subsequently into *E.coli* by chemical transformation. Using ampicillin selection, colonies of *E. coli* containing the vector were grown on agar plates. Multiple (over 50) bacterial colonies were obtained per plate, and 10 colonies (2 from each of 5 agar plates) were isolated and further amplified by growth in ampicillin-containing broth. The resulting DNA was isolated, purified and quantified, then subjected to an Eco-R1 digest to confirm presence of an appropriately-sized insert, representing the cloned HCV 5'UTR sequence. Of the 10 clones, 7 contained a 265-base insert which was visible after electrophoresis on a 1% agarose gel. It was not possible to check the orientation of the PCR product within the plasmid by restriction enzyme analysis as no suitable single cut site in the plasmid and product could be identified. Therefore each sample containing an appropriately-sized insert on Eco-R1 digestion was sent for Sanger sequencing (performed by Source Bioscience, Nottingham, UK) using both T3 and T7 primers. Sequences obtained were aligned with the 5'UTR of the G1a reference sequence H77 and JFH-1 (obtained from the European HCV Database) using BioEdit Sequence Alignment Editor software. Once the correct orientation was established, all seven clones aligned with the 5'UTR region of the G1a reference strain H77, but

not with JFH-1. These results confirmed that output HCV RNA from capture-fusion experiments reflected replication of the input virus, rather than contamination by infectious laboratory strains.

4.4 Quantifiable replication of most viral isolates in the capture-fusion assay

A total of 81 viral isolates were tested in the experiments described in this chapter and in chapter 5. Clinical characteristics of participants are summarised according to genotype in Table 4-1, and listed in full in Appendix 1. HCV RNA could be detected at quantifiable levels (>10 copies/reaction) after fusion in 65 (80%) (Fig 4-6). Of the remaining 16 samples, 10 were archived (3 to 7 years old) and may have degraded during storage. Six other samples produced HCV RNA levels above the level of detection but below the level of quantification in repeated experiments.

Genotype (N)	1 (29)	2 (3)	3 (35)	4 (8)	5 (3)	6 (3)
Number of participants with data available	26 (G1a:G1b 20:6)	3	27 (G3a:G3b 23:4)	3	0	3
M:F	18:8	3:0	16:9	0:3		2:1
Age (median, range)	57 (34-69)	53.5 (49-59)	52 (23-75)	47 (44-48)		51.5 (41-62)
ALT (median, range)	59 (12-280)	127 (22-253)	109 (29-254)	50 (36-88)		80.5 (14-147)
Viral load (x10 ⁶ IU/mL; median, range)	6.28 (4.2-7.19)	6.30 (5.41-6.46)	6.23 (41.9-7.1)	5.45 (3.65 – 5.79)	5.39 (4.22-6.24)	6.22 (5.97-6.38)
Fibrosis score	3 (1-6)	6 (0-6)	5 (1-6)	0		1

Table 4-1. Summary of clinical characteristics of patients with chronic HCV who donated serum samples for capture-fusion experiments. Total number of participants per genotype is given in the top row. The second row refers to the number of participants for whom clinical data was available. Full clinical information was not available for 3 G1 samples supplied by The Royal Free Hospital, 8 G3 samples supplied by Janssen Virology, 5 G4 samples supplied by Dr Hadeel Gamal (Cairo) and 3 G5 samples supplied by King's College Hospital. Viral load was available for all samples. M:F, male:female; ALT, alanine aminotransferase.

4.4.1 Diverse viral genotypes replicate in the capture-fusion assay

Initial experiments were performed using serum from donors infected with G1 and G3 HCV, as these genotypes predominate in the UK. To investigate whether HCV of all known viral genotypes would replicate in the capture-fusion model, serum samples were obtained from patients infected with less common viral genotypes (2, 4, 5 and 6). Samples were tested in the capture-fusion assay, as described in Section 4.3.1, and HCV RNA quantified 5 days after fusion. Although the level of replication varied from patient to patient, there were no consistent differences between genotypes and HCV RNA from isolates of all genotypes could be quantified 5 days after fusion (Fig. 4-6).

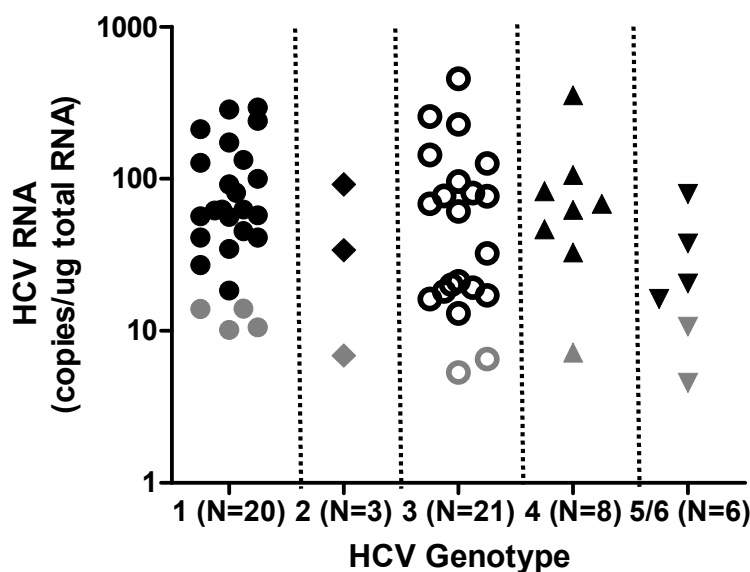


Figure 4-6. HCV RNA can be detected 5 days after fusion in most capture-fusion experiments. HCV RNA was quantified 5 days after fusion in capture-fusion experiments using serum from 58 donors infected with diverse HCV genotypes. Samples which were above the limit of detection but below the level of quantification at day 5 are in grey. Each point represents mean HCV RNA from at least 4 biological replicates per patient sample.

To confirm replication of patient-derived isolates of different genotypes in the assay, fused cells were treated with a range of concentrations of telaprevir. A dose-dependent reduction in viral RNA was seen in capture-fusion experiments with all viral genotypes except G3, which is known to be poorly sensitive to telaprevir. Representative examples are shown in Figure 4-7.

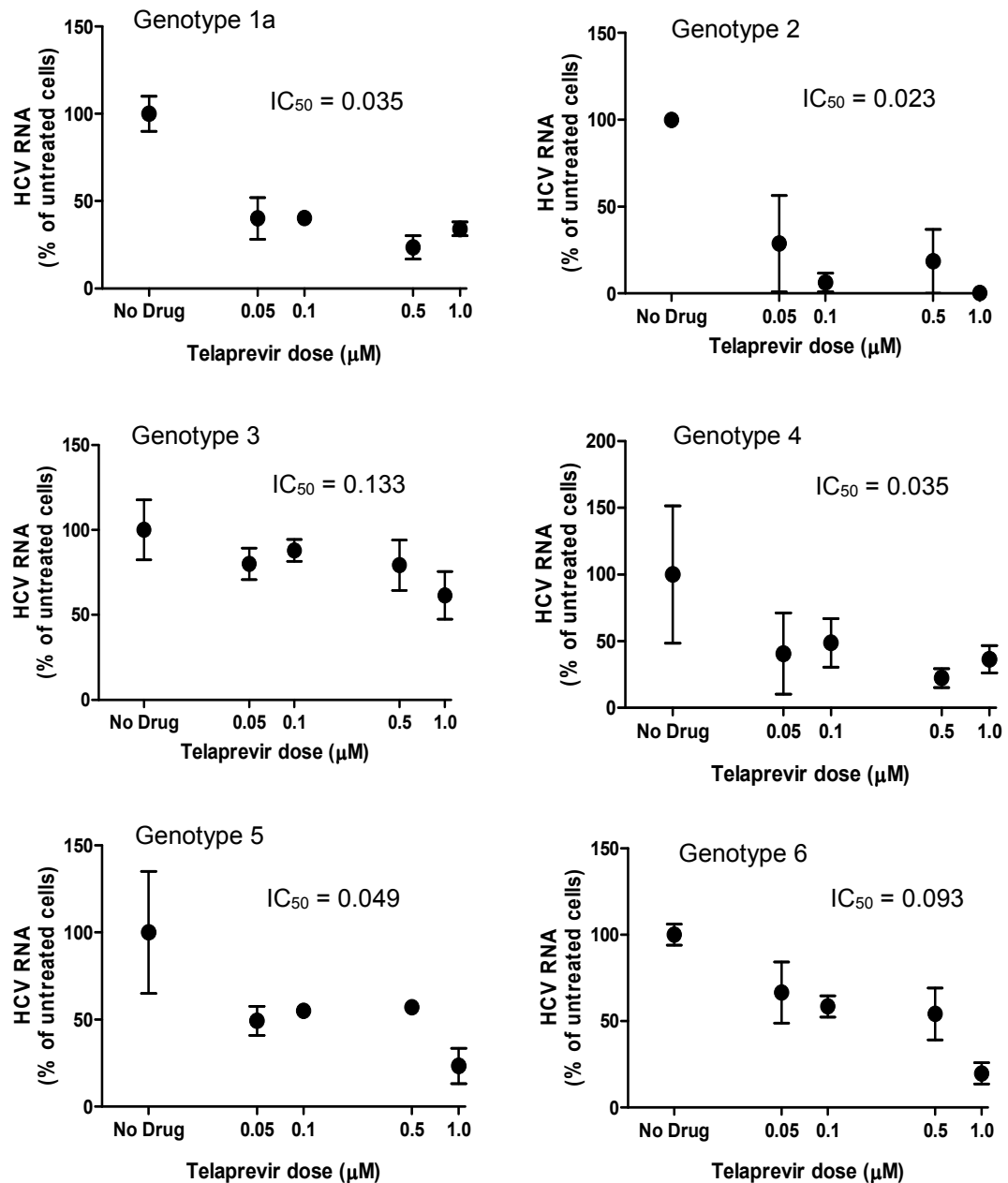


Figure 4-7. Telaprevir inhibited replication of patient-derived HCV of diverse viral genotypes in the capture-fusion assay. In separate experiments, THP-1 cells pre-stimulated with PMA/IFN γ were exposed to serum from patients with HCV genotypes 1-6 prior to fusion with Huh7.5. All G1 samples were from donors infected with G1a. Fused cells were treated in quadruplicate with a range of telaprevir doses for 5 days before HCV RNA quantification. HCV RNA in drug treated wells was calculated as a percentage of that in untreated wells, and dose-response curves constructed for each experiment. These were used to calculate a 50% inhibitory concentration for telaprevir (IC_{50}) for each experiment. Each graph shows mean \pm s.e.m. for an experiment using serum from a single donor infected with each genotype.

4.4.2 HCV replication is independent of clinical features and is consistent for a given sample

HCV RNA yield in the assay varied between samples, but did not relate to donor viral load, fibrosis score or disease activity (Fig. 4-8 A-C).

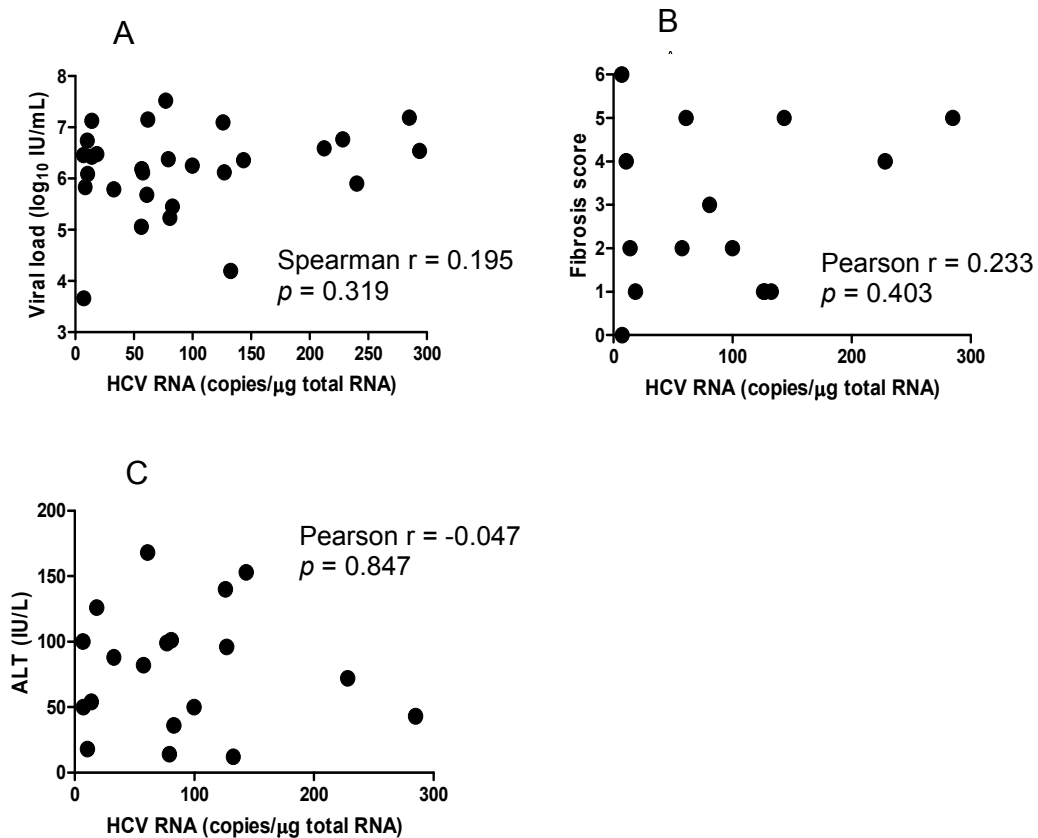


Figure 4-8. HCV RNA yield in the capture-fusion assay is independent of donor clinical features. Replication in the capture-fusion assay did not correlate with donor viral load (A), fibrosis score (B) or ALT (C). Replication is given as mean HCV RNA measured at day 5 post-fusion for each patient. Data are given for all patients where viral load, fibrosis score and ALT were available at the time of sampling. Fibrosis score was measured histologically using the Ishak scoring system. Correlations and p values were calculated using Pearson r correlation coefficient for parametric data or Spearman rank correlation test for non-parametric data.

To address whether the variability in HCV RNA levels seen at day 5 after fusion was due to experimental variation or viral characteristics, capture-fusion experiments were repeated for a number of patients where sufficient serum was available. Variation in HCV RNA yield at day 5 post-fusion was low between repeat experiments, indicating that differences in viral replication between patient samples was influenced by viral characteristics more than experimental variability (Fig. 4-9).

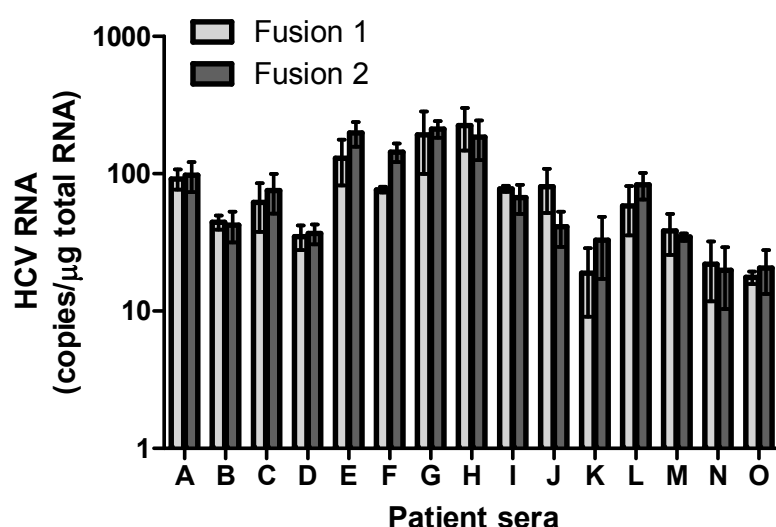


Figure 4-9. Low variation in HCV RNA yield in repeat capture fusion experiments using the same donor serum sample. To measure consistency of HCV RNA yield at day 5 after fusion, capture-fusion experiments were repeated using 15 serum samples where sufficient sample was available. Graph shows mean \pm s.e.m of at least 4 biological replicates per fusion.

4.5 Release of infectious virions after fusion

The experiments described above showed that patient-derived HCV associated with THP-1 cells pre-stimulated with PMA/IFN γ . Evidence suggestive of low-level viral replication was seen after these cells were fused with Huh7.5 cells, specifically an increase in HCV RNA which could be inhibited with antiviral drugs.

Further experiments were performed to investigate whether this model supported full cycles of viral replication with production and release of infectious virions into the supernatant.

As viral replication occurred at low level within the fusion model, it was expected that any virions released into the supernatant would be present at very low copy number. Therefore, supernatants from multiple wells (1mL supernatant per well, total volume 10-20 mL) were collected and concentrated by centrifugation on a sucrose gradient (details in chapter 2). Direct RT-qPCR analysis of concentrated supernatant confirmed the presence of small amounts of HCV RNA. A method to test infectivity of the supernatant was sought, to establish whether the presence of HCV RNA equated to presence of infectious virions. Unlike cell culture produced HCV, patient-derived strains do not infect Huh7.5 cells (Fig. 4-4). However it is possible that after cell fusion, viral replication in the fused cells may have selected for a strain capable of *de novo* infection of Huh7.5. To investigate whether capture-fusion supernatants contain infectious virions, a further cycle of capture-fusion experiments was performed, using concentrated supernatant in place of patient serum. In two independent experiments using concentrated supernatants from capture-fusion experiments with serum from a G1 and a G3 donor, an increase in viral RNA was seen after fusion of THP-1 cells infected with the concentrated supernatant indicating replication of viable HCV (Fig. 4-10A, B). Sufficient concentrated supernatant was available from the original G3 capture-fusion experiment to also inoculate directly onto Huh7.5 cells for 24 hours. The cells were then washed thoroughly and HCV RNA quantified up to 5 days after infection. In this single experiment, there was a small but significant increase in

HCV RNA quantifiable from Huh7.5 exposed to concentrated supernatant (Fig 4-10B), suggesting that virions present in the capture-fusion supernatant may have successfully entered fresh Huh7.5 cells and established replication, albeit at very low level.

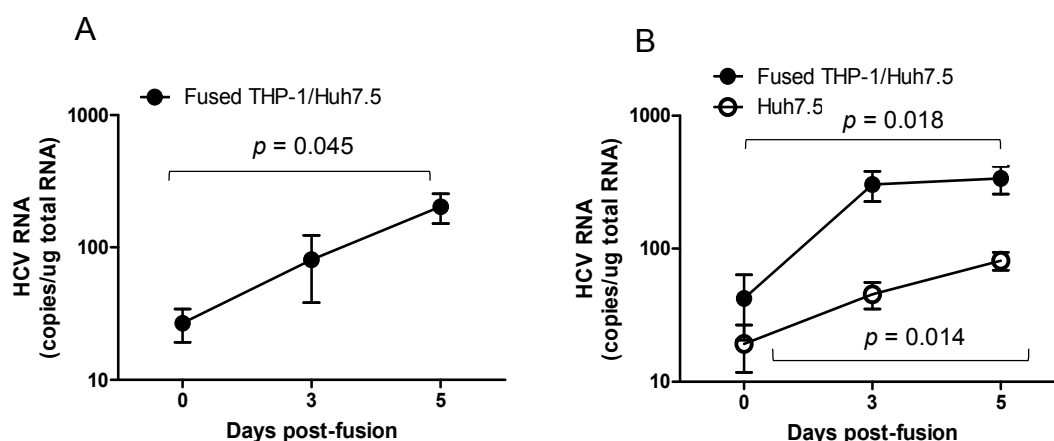


Figure 4-10. Infectious virions are released into capture-fusion supernatants. Results of experiments using concentrated supernatants from 2 separate capture-fusion experiments, one using a serum from a G1 HCV infected donor (**A**) and the other from a G3 HCV infected donor (**B**). Concentrated supernatant was applied to pre-stimulated THP-1 cells for 24 hours, before fusion with Huh7.5 and quantification of HCV RNA immediately after fusion and after 3 and 5 days in culture. (**B**) also shows results of inoculation of concentrated supernatant directly onto fresh Huh7.5 cells for 24 hours. After washing, HCV RNA was quantified immediately then 3 and 5 days after infection. In (**A**), $p = 0.045$ for comparison between day 0 and day 5 post-fusion. In (**B**), $p = 0.018$ for the comparison between fused cells at day 0 and day 5 post-fusion, and $p = 0.014$ for the comparison between infected Huh7.5 at day 0 and day 5 post-infection. Graphs show mean \pm s.e.m. Graphs each show results from a single capture-fusion experiment. P values were calculated using Mann-Whitney U test.

4.6 Mechanisms underlying HCV entry and replication in the capture-fusion assay

The experiments above showed that patient-derived HCV could be “captured” from patient serum by pre-stimulated THP-1 and induced to replicate following fusion with Huh7.5 cells. Full replicative cycles appeared to occur, with release of infectious virions into the supernatant. Although in the majority of experiments

the level of HCV RNA detectable after cell fusion was above the quantification limit of the sensitive PCR assay and could clearly be inhibited with antiviral drugs, the low level of viral replication made studies on the mechanisms underlying viral entry and replication difficult. To address this, HCV pseudoparticles (HCVpp) were employed to study entry of HCV into THP-1 cells and capture-fusion experiments were performed using cell culture HCV (HCVcc) in an attempt to achieve high level replication after fusion.

4.6.1 Cell culture HCV (HCVcc) does not replicate in the capture-fusion assay

The capture-fusion assay was performed using infectious supernatants containing the HCV cell culture strain JFH-1. JFH-1 replicates to high levels in Huh7.5 cells [28, 99], and would facilitate mechanistic studies in contrast to the low-level replication seen using patient-derived HCV. THP-1 were stimulated with PMA/IFN γ then incubated with JFH-1, the infectivity of which had previously been titrated on Huh7.5 cells (see chapter 2). After 24 hours incubation, JFH-1 associated with both unstimulated and PMA/IFN γ stimulated THP-1. Unlike infection with patient-derived HCV, PMA/IFN γ did not significantly enhance association of JFH-1 with THP-1 (Fig. 4-11A). When THP-1 were incubated with JFH-1 then fused with Huh7.5 cells, a decline in HCV RNA was seen up to 5 days after fusion, implying a lack of JFH-1 replication in fused cells. This was seen whether THP-1 were stimulated with PMA/IFN γ prior to JFH-1 exposure or not (Fig. 4-11B). Furthermore, indirect immunofluorescence found no evidence of HCV protein production in THP-1 exposed to JFH-1 before or after fusion with Huh7.5 (data not shown).

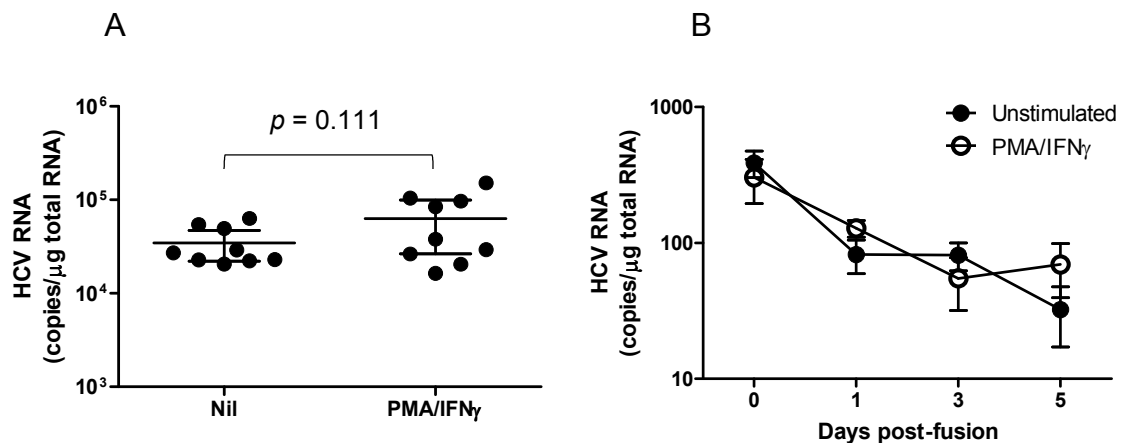


Figure 4-11. PMA/IFN γ stimulation did not enhance association of JFH-1 with THP-1 or replication after fusion with Huh7.5. **A.** THP-1 cells, treated with PMA (200ng/mL) and IFN γ (10ng/mL) or with no stimulants for 24 hours, were incubated with JFH-1 (0.5 MOI (multiplicity of infection) per cell) for 24 hours, before quantification of HCV RNA (**A**). These THP-1 were then fused with Huh7.5 cells and HCV RNA quantified immediately after fusion, and after 3 and 5 days (**B**). $P = 0.111$ mean \pm 95% CI. Graph shows results from a single representative experiment. P value was calculated using Mann Whitney U test.

4.6.2 HCV pseudoparticles (HCVpp) do not infect THP-1

HCVpp comprise functional HCV envelope glycoproteins assembled with a luciferase reporter onto retroviral or lentiviral core particles [88]. They are not capable of replication but have been used extensively to study entry of HCV into hepatocytes (reviewed in [291]). In experiments performed by Dr Jenny Waters infection of THP-1 cells by HCV pseudoparticles could not be achieved (data not shown). These data suggest that viral entry mechanisms in monocytes may differ from those in hepatocytes.

4.7 Patient-derived HCV does not utilise classical entry receptors for attachment/entry to THP-1

Entry of HCVcc and HCVpp into hepatocytes is dependent upon CD81, SR-B1 and the tight junction proteins claudin-1 and occludin (see for example [22, 26, 30, 32, 37, 38]). Neither HCVpp nor HCVcc infected THP-1 and PMA/IFN γ treatment of THP-1 enhanced association of patient-derived HCV but not HCVcc, suggesting that HCV entry into monocytes may use different receptors, perhaps scavenger receptors such as Fc γ receptors. We investigated expression of these receptors on THP-1 cells and their potential role in uptake of patient-derived HCV in the capture-fusion model.

4.7.1 THP-1 expression of CD64, but not classical HCV entry receptors, is upregulated by PMA/IFN γ

Association of patient-derived HCV, although not HCVcc, with THP-1 cells was enhanced by pre-stimulation of THP-1 with PMA/IFN γ . To examine whether PMA/IFN γ pre-stimulation upregulated expression of classical HCV entry receptors or Fc γ R on THP-1 cells, mRNA expression of CD81, SR-B1, LDL-R, CD64 (Fc γ IR), CD32 (Fc γ IIIR) and CD16 (Fc γ IIIR) was measured by real time PCR before and at various times after stimulation of THP-1 cells with PMA and IFN γ . Claudin-1 and occludin mRNA was not detected in unstimulated or stimulated THP-1 cells (data not shown). This was expected, as these cells do not form tight junctions. CD16 mRNA was not detected either before or after PMA/IFN γ stimulation (data not shown). CD81, SR-B1 and LDL-R mRNA was expressed by THP-1 cells at baseline but expression declined over 24 hours after PMA/IFN γ stimulation (Fig 4-12 A-C). CD32 and CD64 were expressed at

baseline but only expression of CD64 increased after PMA/IFN γ stimulation (Fig 4-12 D, E).

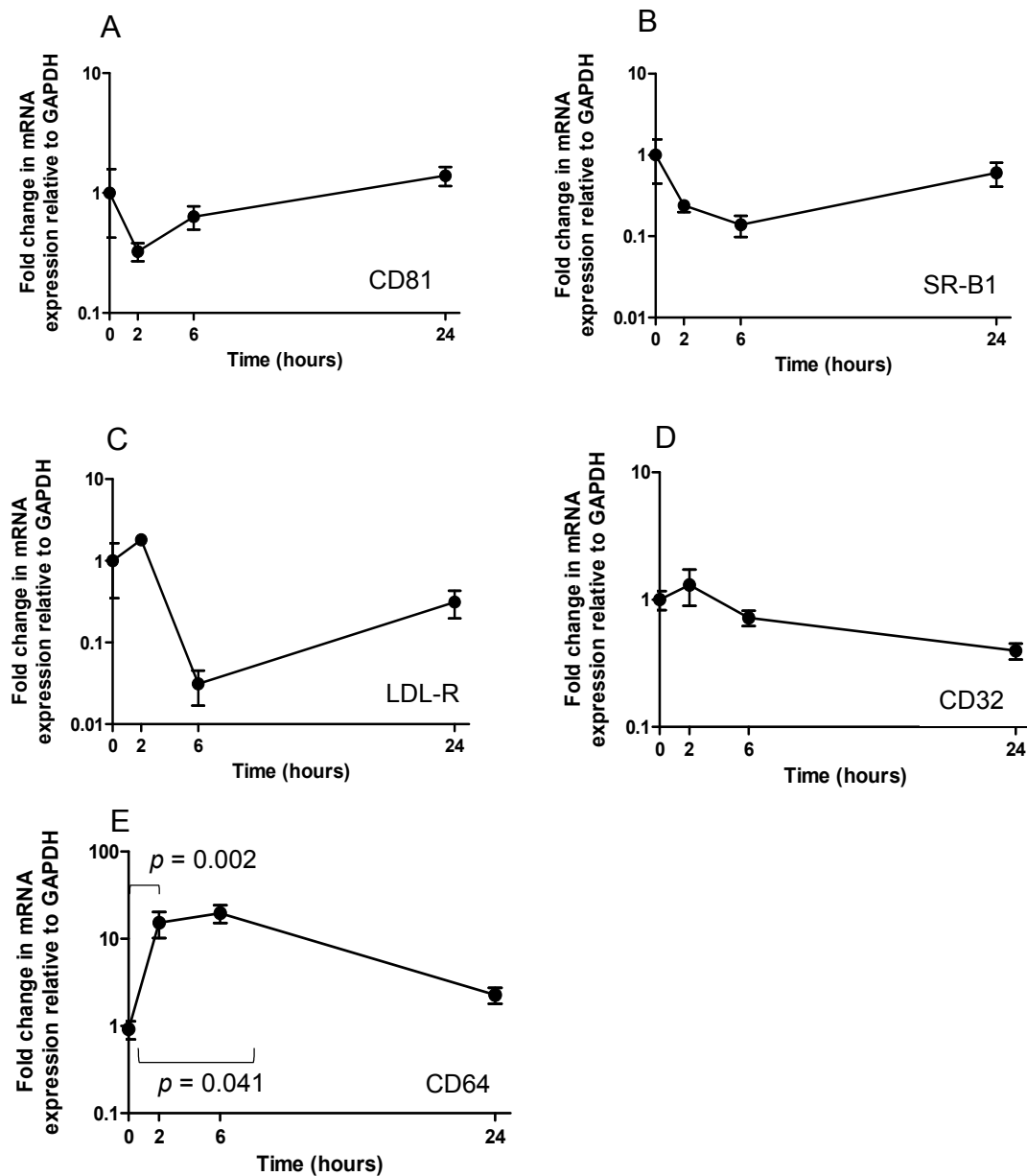


Figure 4-12. Expression of CD64, but not of other candidate receptors for HCV entry, is increased by PMA and IFN γ stimulation of THP-1 cells. mRNA expression of classical HCV entry receptors CD81 (A), SR-B1 (B) and LDL-R (C) and of Fc γ receptors CD32 (Fc γ IIIR, D) and CD64 (Fc γ IR, E) on THP-1 cells over 24 hours after stimulation with PMA (200ng/mL) and IFN γ (10ng/mL). mRNA is expressed relative to GAPDH and to unstimulated cells. CD16 (Fc γ RIII), claudin-1 and occludin mRNA could not be detected in stimulated or unstimulated cells. Graphs show mean \pm s.e.m. of results from 2 independent experiments. *P* values were calculated using Student's *t*-test.

Protein expression is regulated at multiple levels. To confirm that the observed increase in CD64 mRNA expression translated into an increase in receptor expression, cell surface CD81, SR-B1, CD32 and CD64 were quantified by flow cytometry. Receptor expression was compared on unstimulated THP-1 and after stimulation with PMA or PMA and IFN γ for 24 hours. CD81 and CD32 surface expression was not significantly increased by treatment with either PMA or PMA/IFN γ (Fig 4-13 A, B). CD64 expression, however, increased after PMA treatment and a synergistic effect of PMA and IFN γ stimulation was observed (Fig 4-13 C). Technical problems made quantification of SR-B1 expression by flow cytometry difficult as the only commercially available antibody was a polyclonal rabbit anti-human SR-B1. The isotype control for this antibody gave a high background signal which made it difficult to detect a true increase in protein expression. However, no clear increase in SR-B1 was seen after either PMA or PMA/IFN γ stimulation, in keeping with the results of the mRNA expression experiments and further suggesting that SR-B1 was not significantly upregulated by PMA or PMA/IFN γ treatment. CD64 was the only candidate receptor tested which was upregulated at both the mRNA and cell surface level by treatment with PMA/IFN γ . Given that PMA/IFN γ also enhanced association of patient-derived HCV with THP-1 cells, this suggested that CD64 might play a role in attachment or entry of patient-derived HCV into THP-1.

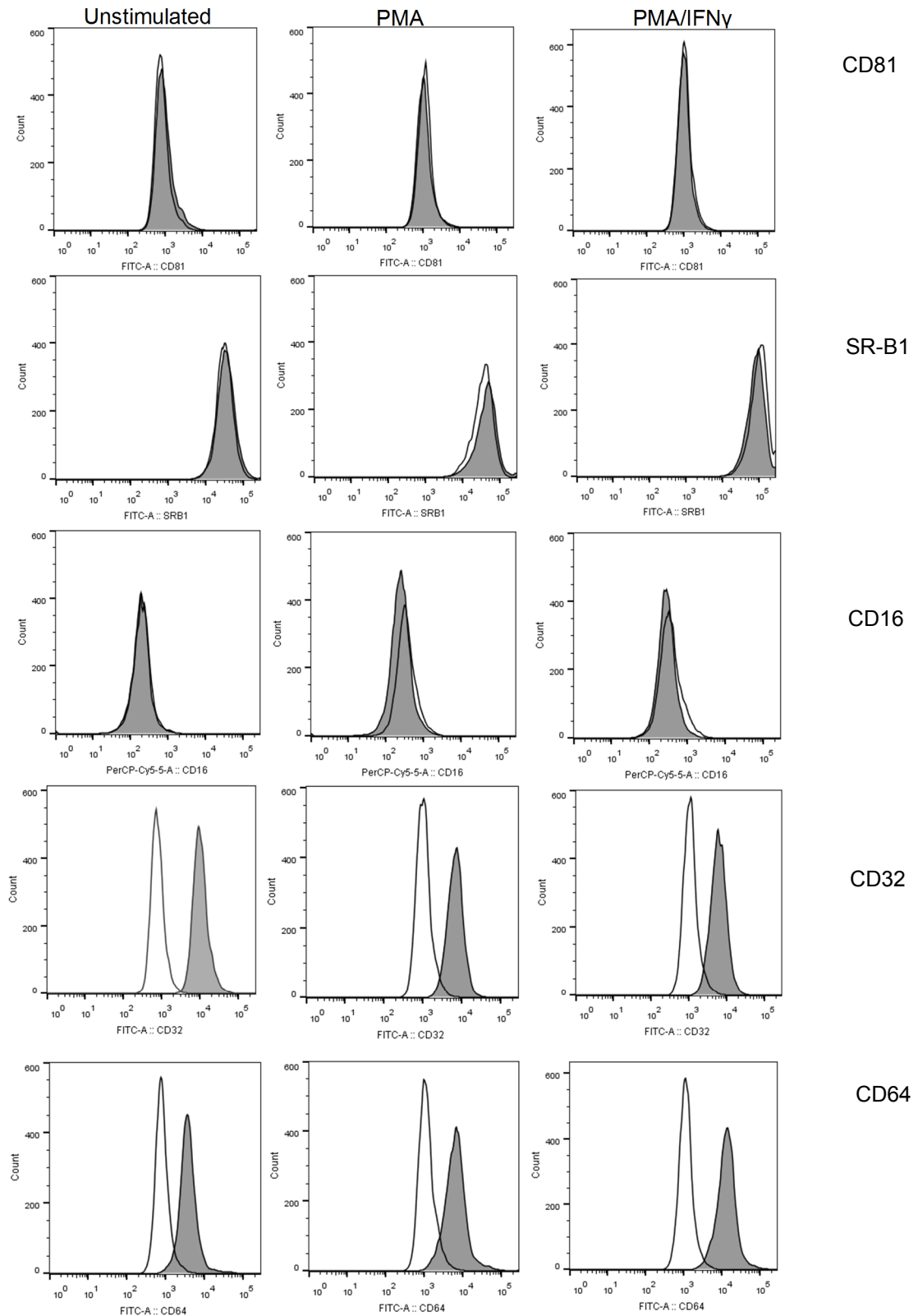


Figure 4-13. Expression of CD64, but not CD81 or CD32, is upregulated on THP-1 by PMA and IFN γ . THP-1 cells were incubated with PMA 200ng/mL, PMA 200ng/mL and IFN γ 10ng/mL or with no stimulants for 24 hours before fixation and staining with antibodies to CD81, SR-B1, CD16, CD32, CD64 or isotype controls. Receptor expression was quantified by flow cytometry. Grey histograms show receptor expression, clear histograms show isotype control.

4.7.2 Blocking CD64 reduces HCV replication after fusion

To investigate the role of CD64 in THP-1 attachment/entry of patient-derived HCV which replicated after fusion with Huh7.5 cells, PMA/IFN γ stimulated THP-1 were incubated with anti-CD81 or anti-CD64 blocking antibodies at a range of concentrations prior to exposure to patient serum. Association of patient-derived HCV with stimulated THP-1 was not significantly reduced by blocking of CD64 or CD81 (which is classically associated with HCV entry into hepatocytes) (Fig 4-14A). However HCV yield after fusion was reduced when THP-1 cells were pre-incubated with CD64 blocking antibodies before infection and fusion with Huh7.5 cells, but not when the THP-1 were pre-incubated with anti-CD81 antibodies (Fig. 4-14B, C). Taken together, these results suggest that uptake of patient-derived HCV into THP-1 cells may be mediated at least in part by CD64. Uptake via this receptor permits replication to occur after fusion with Huh7.5 cells. Blocking of CD64 does not abrogate association of HCV with THP-1 cells, suggesting that additional receptor(s) may play a role, however this does not appear to be via the classical CD81 pathway associated with HCV entry into hepatocytes.

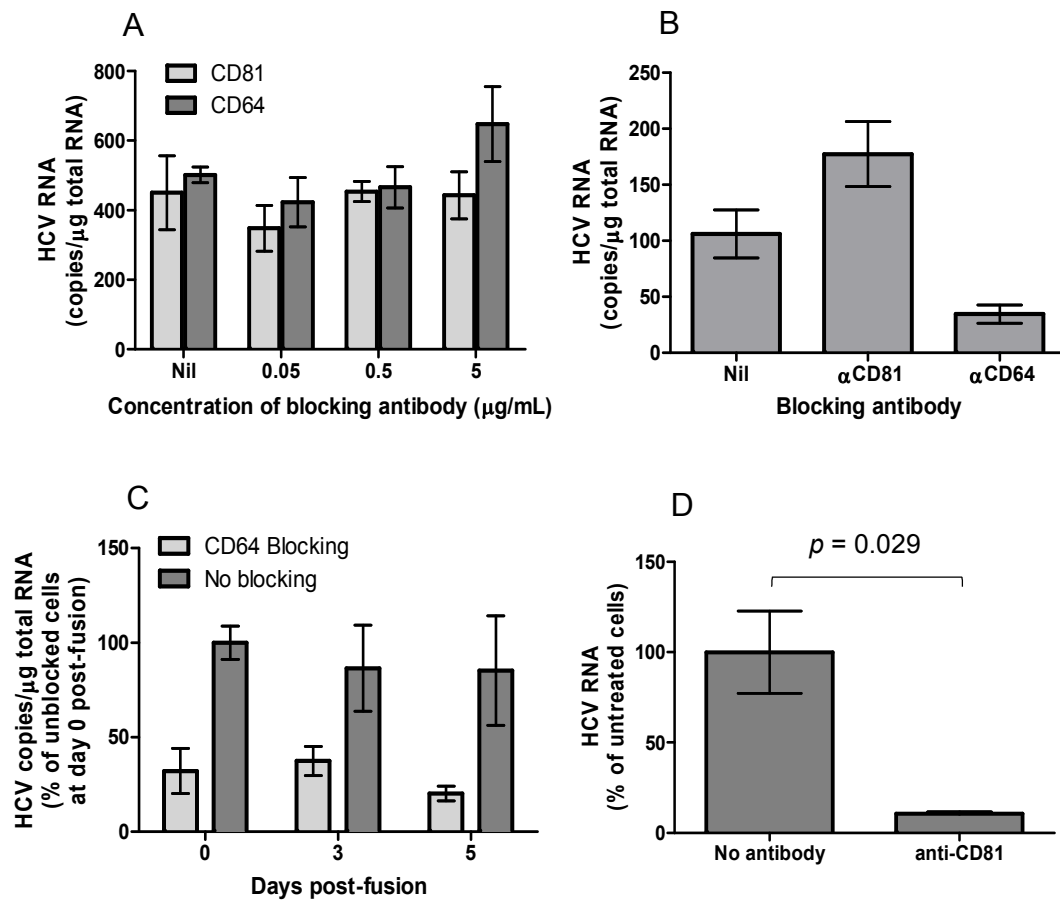


Figure 4-14. CD64 blocking does not reduce attachment of patient-derived HCV to THP-1 but does reduce replication after fusion with Huh7.5 cells. **A**, pre-stimulated THP-1 were incubated with anti-CD81 or anti-CD64 blocking antibodies at a range of concentrations for 1 hour, prior to exposure of the cells to HCV (+) patient sera for 24 hours then quantification of HCV RNA. Graph shows mean \pm sem of 3 independent experiments. **B**, in a further experiment, pre-stimulated THP-1 were incubated with anti-CD81 or anti-CD64 antibodies (5μg/mL), prior to incubation with patient serum then fusion with Huh7.5 cells. HCV RNA was quantified immediately after fusion. **C**, in one further experiment, pre-stimulated THP-1 were incubated with anti-CD64 antibody (5μg/mL), prior to incubation with patient serum then fusion with Huh7.5 cells. HCV RNA was quantified immediately, then at 3 and 5 days after fusion. **D**, control experiment to confirm efficacy of the CD81 blocking antibody. Huh7.5 cells were incubated in the presence or absence of anti-CD81 blocking antibody (5μg/mL) for 1 hour before infection with the HCV cell culture strain JFH-1. HCV RNA was quantified 72 hours after infection and expressed as a percentage of cells infected in the absence of blocking antibody. *P* value was calculated using Mann-Whitney U test.

4.7.3 Anti-HCV antibodies do not enhance uptake of HCVcc into THP-1

If entry of patient-derived HCV into THP-1 is mediated through FcγR, specifically CD64, then it is possible that anti-HCV antibodies forming antibody/viral

complexes may play a role in viral uptake into monocytes. This hypothesis may explain why cell culture-derived virus (JFH-1) is not active in the 'capture-fusion' model. To examine this we assayed JFH-1 infectivity after incubation with sera from patients with past HCV infection. Sera were available from a panel of donors who had cleared HCV infection. These HCV RNA (-), anti-HCV IgG (+) sera were screened for reactivity to JFH-1 protein using an established ELISA technique [270] (Chapter 2). Samples with the highest reactivity to JFH-1 were used for subsequent experiments. In 2 independent experiments, JFH-1 was incubated with each serum at a range of dilutions, before incubation with PMA/IFN γ stimulated THP-1 cells for 24 hours and then fusion with Huh7.5. Incubation with sera containing anti-HCV antibodies did not enhance JFH-1 association with THP-1 cells or replication after fusion with Huh7.5 (Fig. 4-15).

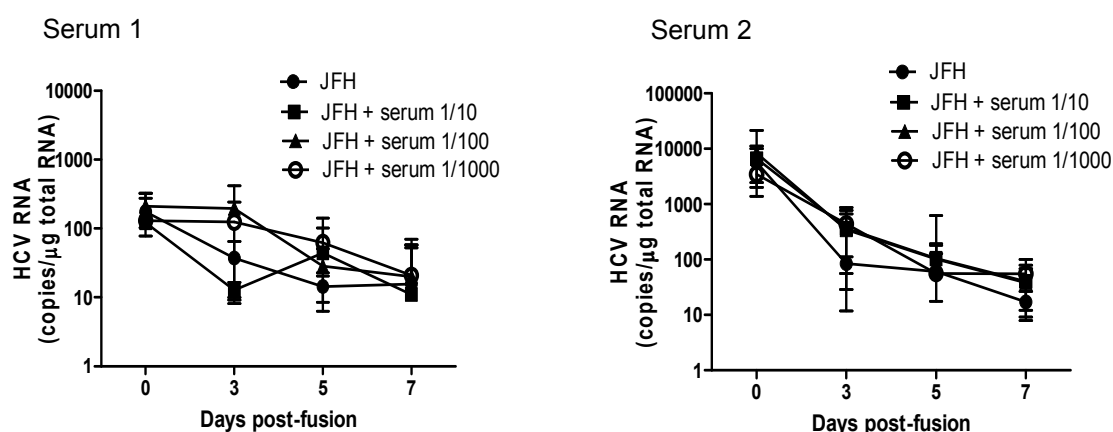


Figure 4-15. JFH-1 association with THP-1 and replication after fusion was not enhanced by anti-HCV antibodies. JFH-1 was incubated with HCV (-), anti-HCV (+) sera from 2 patients, which were reactive to JFH-1, at a range of dilutions before incubation with THP-1 cells. The THP-1 were then fused with Huh7.5 cells and HCV RNA measured at various times after fusion. Graphs show mean \pm sem for each individual experiment.

4.7.4 Cell fusion does not trigger expression of interferon stimutable genes

We hypothesised that the fusion process permits transfer of HCV to Huh7.5 cells without triggering induction of interferon and innate antiviral responses. However the fused cells remain interferon sensitive, as exogenous interferon inhibited replication of interferon sensitive viral strains (Fig. 4-5 and Chapter 5). We compared expression of the interferon stimutable gene MxA in fused cells with and without HCV exposure and with and without interferon treatment. We found that MxA expression is not induced by cell fusion and is low in infected cells, but can be upregulated by exogenous interferon (Fig. 4-16A). Furthermore, priming of Huh7.5 cells with interferon significantly reduced HCV replication after fusion (Fig. 4-16B). These data suggest that at least one of the mechanisms underlying the capture-fusion replication process is delivery of virus to hepatocytes without triggering an intracellular innate immune response.

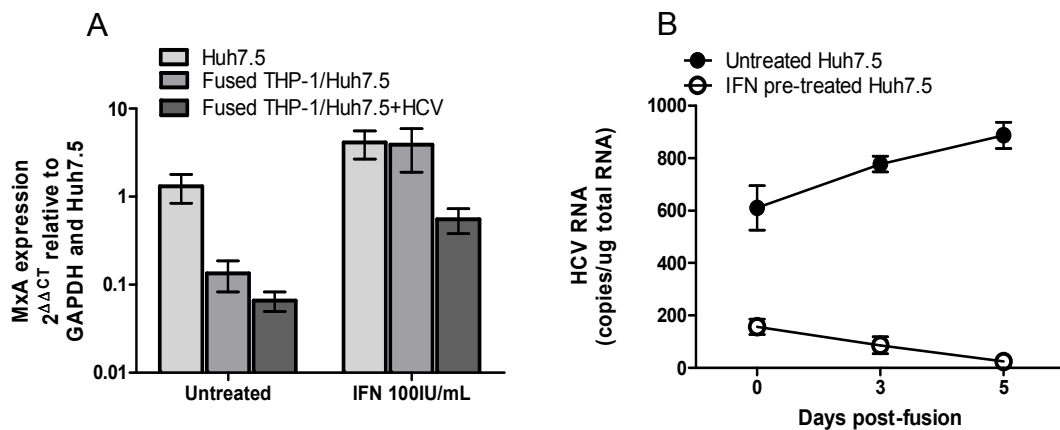


Figure 4-16. The fusion process does not trigger expression of the interferon stimutable gene MxA, but MxA expression remains inducible by exogenous IFN. **A**, fused HCV-infected THP-1/Huh7.5, fused uninfected THP-1/Huh7.5 or unfused Huh7.5 were cultured for 5 days in the presence or absence of IFN α -2a (100 IU/mL). MxA mRNA was quantified by PCR and expressed relative to GAPDH and uninfected, unfused, untreated Huh7.5. Results are shown on a logarithmic scale. **B**, Untreated Huh7.5 cells or Huh7.5 pre-treated with 100 IU/mL IFN α -2a for 1 hour were fused with THP-1 cells infected with patient serum. HCV RNA was quantified immediately and at 3 and 5 days after fusion. Each graph shows mean \pm s.e.m. of a single representative experiment.

4.7.5 Fusion with THP-1 does not render Huh7.5 more permissive to HCV replication

It is conceivable that the process of cell fusion results in release of factor(s) which enhance permissiveness of Huh7.5 to HCV entry and/or replication. To establish whether this was the case, an equal number of PMA/IFN γ pre-stimulated THP-1 cells and Huh7.5 cells were stained with the fluorescent membrane dyes Dil (THP-1) or DiO (Huh7.5) prior to fusion. After fusion, dual labelled cells were sorted by fluorescence activated cell sorting (FACS) to identify the fused THP-1/Huh7.5 population and cultured overnight. In parallel, an equivalent number of dye-labelled Huh7.5 and THP-1 cells were cultured together without fusion. The fused or co-cultured cells were infected with the HCV cell culture strain JFH-1 and HCV RNA quantified after 72 hours. No enhancement of JFH-1 replication was seen in the fused cells compared to the co-cultured cells (Fig. 4-17), excluding fusion-induced enhancement of Huh7.5 permissiveness as a mechanism underlying replication after fusion.

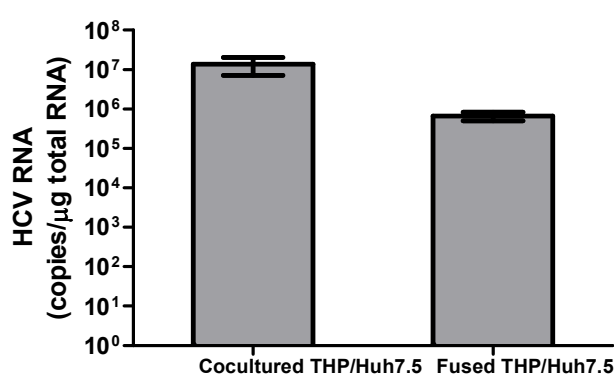


Figure 4-17. Fusion with THP-1 does not render Huh7.5 cells more permissive to HCV replication. Pre-stimulated THP-1 and Huh7.5 were stained with fluorescent membrane dyes and prior to fusion. After fusion, dual-labelled cells were isolated by FACS and infected with JFH-1 at 0.5 MOI/cell (fused THP/Huh7.5). An equal number of labelled, unfused THP-1/Huh7.5 (cocultured THP/Huh7.5) were also infected at the same multiplicity of infection. After 72 hours HCV RNA was quantified. Graph shows mean \pm s.d. of biological quadruplicates in a single experiment.

4.8 Enhancement of HCV replication in the capture-fusion model

Similar to HCV replication seen after fusion of patient-derived monocytes with Huh7.5 cells, the level of replication of patient-derived HCV in the capture-fusion assay was low, often near the limit of quantification of the sensitive RT-qPCR assay. In an effort to improve consistency and reliability of results, attempts were made to enhance HCV replication after fusion.

4.8.1 Activation of the sonic hedgehog signalling pathway

Others have shown that upregulation of the hedgehog signalling pathway enhances replication of cell culture produced HCV (HCVcc) [292]. However, we found no enhancement of replication of patient-derived HCV after treatment of fused cells with the hedgehog agonist SAG (data not shown). Osteopontin, a profibrogenic extracellular matrix protein and cytokine, is upregulated by activation of the hedgehog signalling pathway and also enhances replication of HCVcc [293, 294]. Osteopontin treatment of fused THP-1/Huh7.5 cells showed a trend towards enhanced replication of patient-derived HCV at the highest concentrations used (Fig 4.18A), although this was not statistically significant. Others have reported that OPN-mediated enhancement of HCV replication is greater in Huh7 cells than in Huh7.5 as baseline OPN levels are higher in Huh7.5 cells [293]. Where fusion experiments were performed with Huh7 cells, HCV RNA yield was enhanced by OPN in 2 out of 3 patient samples tested (Fig 4-18B).

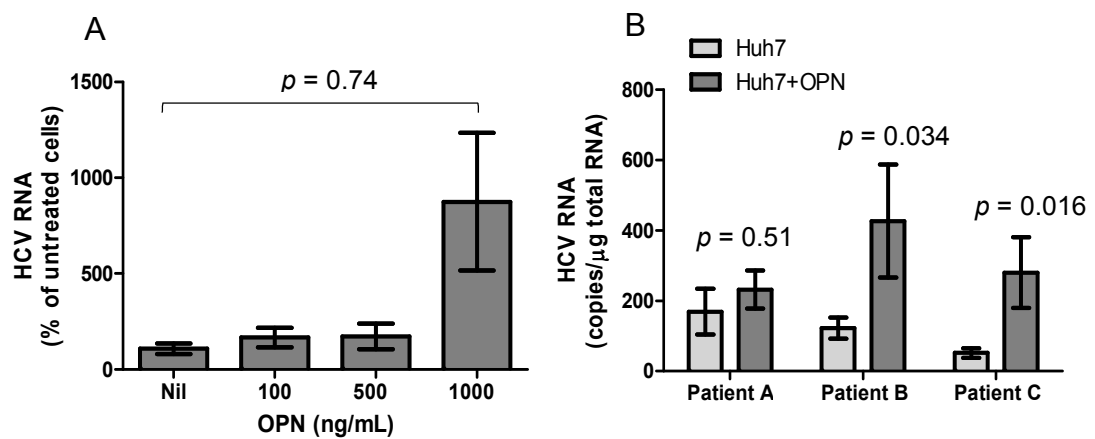


Figure 4-18. Effect of osteopontin (OPN) treatment on HCV replication in fused cells. A, mean \pm sem of results from 2 independent experiments where prestimulated, HCV-exposed THP-1 fused with Huh7.5 cells were treated with osteopontin (OPN) at a range of concentrations for 5 days after fusion, before quantification of HCV RNA. **B** shows results from a further 3 independent experiments in which prestimulated, HCV-exposed THP-1 were fused with Huh7 cells then cultured in the presence or absence of OPN (1000ng/mL) for 5 days before quantification of HCV RNA. p values were calculated using Mann-Whitney U test.

4.9 Discussion

Following our demonstration that patient-derived HCV replicates after fusion of patient monocytes with Huh7.5 cells, we hypothesised that monocytic cell lines could be used to transmit virions from patient serum samples to Huh7.5 cells and induce viral replication in a similar manner. The monocytic cell line THP-1 was readily available in the laboratory and was evaluated in these experiments. Alternate monocytic cell lines were either found to be unsuitable for use in this assay due to proliferation and clumping in culture (MonoMac1) or were subsequently evaluated and found to provide no additional benefit in terms of HCV yield over THP-1 (U937, experiments performed by Dr Meleri Jones, data not shown).

Replication of HCV derived from patient serum after “capture” by pre-stimulated THP-1 cells and fusion with Huh7.5 was demonstrated by accumulation of HCV RNA over time after fusion, inhibition of RNA accumulation by antiviral drugs, and demonstration of HCV protein production. The vast majority of patient samples could be induced to replicate after fusion, regardless of viral genotype, although analysis of uncommon viral genotypes (particularly G5 and G6) was limited as these genotypes are very rare in the UK. Of the samples which did not replicate in the assay, some may have been due to degradation of archived viral RNA, but a few freshly taken samples did not replicate in repeated capture-fusion experiments. Amongst samples which did replicate, the levels of replication were consistent in repeated capture-fusion experiments for a given sample. These findings suggest that most viral isolates will replicate in this assay, but replication levels vary between samples and this is predominantly determined by intrinsic viral characteristics, rather than experimental variation.

Establishing whether patient-derived HCV underwent full replicative cycles in the capture-fusion model, with release of infectious virions into the supernatant, proved challenging. This was partly due to the low level of viral replication seen (and therefore extremely low levels in experimental supernatants), and also as patient-derived viral strains do not directly infect cultured cell lines, making the use of standard viral infectivity assays impossible. To address the issue of low viral yield supernatants were concentrated on a sucrose gradient. In an attempt to establish whether the concentrated supernatants contained infectious virions, the capture-fusion assay was employed again to assay viral replication, using concentrated supernatant in place of patient serum. In 2 separate experiments,

HCV from concentrated supernatant appeared to accumulate over time after capture by THP-1 and fusion with Huh7.5. Interestingly, accumulation of HCV RNA was also seen after exposure of naïve Huh7.5 cells to concentrated supernatants, although at a lower level than that seen in the fused cells. This raises the possibility of generation or selection of a viral strain adapted for replication in this culture environment. Unfortunately in a single round of experiments the HCV RNA levels achieved were too low for cloning and sequencing of the viral product, but it is possible that this might be enhanced by further round of replication in the capture-fusion assay and this remains an area for future work.

Association of HCV from patient serum with THP-1 was greatly enhanced by pre-treatment of cells with PMA and IFN γ . A proportion of virus (approximately 20%) appeared to be internalised into the cells, as this was resistant to trypsin treatment of the cells from 4 hours after exposure to patient serum. However, we found no evidence to support replication of HCV within THP-1. In keeping with the findings of others, we were unable to infect THP-1 with cell culture produced HCV (JFH-1), or with HCVpp [122]. This made further study of HCV entry into THP-1 difficult, as it was reliant on relatively low-level HCV association with the cells, further confounded by sample-sample variability. In addition, JFH-1 was not efficiently transmitted in the capture-fusion assay, complicating investigation of mechanisms underlying replication following fusion of THP-1 with Huh7.5. HCV protein production could not be reliably detected in fused cells, most likely due to non-specific antibody binding by THP-1 cells. This means that more detailed investigation, such as subcellular localisation of HCV replication, was not

achievable. The development of sensitive fluorescence-emitting constructs which can be used to track HCV replication within the cell in real time may help to address this [295], and these have recently been obtained in the lab.

The enhancement of HCV association with THP-1 cells by PMA and IFN γ was marked and consistent. This was initially unexpected, as IFN γ is a component of the innate anti-viral immune response, and as such might be reasonably expected to reduce viral association or persistence/replication after fusion. However, others have described enhancement of Fc γ receptor (Fc γ R) expression on monocytic cell lines by IFN γ [123, 296-298]. We speculated that as patient-derived HCV associated with THP-1 cells after PMA/IFN γ treatment, but cell culture produced strains did not, Fc γ R may be upregulated by PMA/IFN γ and may be responsible for this association. Previously, CD32 (Fc γ IIIR) was shown to be upregulated on U937 cells after PMA/IFN γ , and this was associated with increased attachment of HCV-Ig complexes to these cells [123]. Upon exposure of THP-1 to PMA/IFN γ , we found no change in CD32 expression, but expression of CD64 (Fc γ IR) was enhanced at the mRNA and cell surface level, more so by PMA/ IFN γ than by PMA alone. Interestingly, pre-exposure to CD64 blocking antibodies did not reduce HCV association with stimulated, unfused THP-1, but a reduction was seen after the fusion step had been performed, and less HCV RNA accumulation was seen after fusion where THP-1 had been exposed to CD64 blocking antibodies before incubation with patient sera. However, entry of JFH-1 into THP-1 and replication of JFH-1 after fusion could not be enhanced by pre-incubation with patient sera containing anti-HCV antibodies. This suggests that although CD64 may play a role in uptake of patient-derived virus and

replication after fusion, other receptors are also involved. Alternatively, the range of mechanisms by which patient-derived virus interacts with THP-1 may be different from those of JFH-1 at a number of levels which are not fully explored in these experiments. Other candidate receptors which warrant further investigation are the lectins L-SIGN and DC-SIGN, which have been implicated in attachment of HCVcc to B cells [130] and also in protection of HCV virus-like particles from lysosomal degradation after uptake into THP-1 cells [299]. The role of exosomes in transfer of cellular material, including viral RNA, is gaining increasing recognition, and this represents another potential mechanism for viral infection of cells [300-302].

A major limiting factor in these experiments, especially in attempts to explore mechanisms underlying the success of the 'capture-fusion' strategy, is the very low level of viral replication observed in this model. The Huh7 cell line and its derivatives, including Huh7.5 cells, are unique amongst hepatoma cell lines in their permissiveness to replication of highly adapted HCV replicons and cell culture strains. Replication depends on acquisition of cell culture adaptive mutations, and with the exception of the highly atypical viral strain JFH-1, viral strains without adaptive mutations cannot be propagated in tissue culture [28, 90-92, 99]. The properties conferred by such mutations, and why they permit replication in this cell line, is incompletely understood. The Huh7.5 cell line was derived from Huh7 by identification of a highly permissive Huh7 subclone which was cured of HCV replicon infection with interferon [276]. The attributes of the Huh7.5 line which make it more permissive to HCV replication are incompletely understood, but are thought to include defective RIG-I signalling [285]. Clearly,

however, these cells remain responsive to exogenous interferon, demonstrated both by the method by which they were generated and response to IFN in capture-fusion experiments. In a limited number of experiments we conducted using Huh7 cells, we found overall lower levels of HCV RNA than in experiments using Huh7.5 cells, suggesting the difference in permissiveness extend to patient-derived HCV in the capture-fusion model as well as to the highly adapted replicon and atypical HCVcc strains.

Both enhancement and inhibition of HCV replication by the profibrogenic extracellular matrix protein and cytokine osteopontin (OPN) have been reported in published literature [293, 303]. Interestingly, OPN has also been reported to enhance replication of HIV in primary monocyte-derived macrophages [304]. Our observations suggest that OPN may enhance replication of patient-derived HCV in the capture-fusion model, particularly when less permissive cell lines are used, but some viral strains may be more susceptible than others. Whether OPN “boosting” is likely to be a useful strategy to enhance viral replication in the capture-fusion assay will require a more detailed understanding of the interactions between OPN and HCV which facilitate replication.

5.0 Results: validation of the “capture-fusion” assay to measure drug sensitivity of patient-derived HCV

5.1 Introduction

We have demonstrated that patient-derived HCV of diverse viral genotypes can be induced to replicate in the capture-fusion model, and can be inhibited with antiviral drugs, suggesting that this assay could be used to assess patient-specific sensitivity to current and novel antiviral therapies. At present, *in vitro* models to test HCV drug sensitivity are limited to the genotype (G)1-based replicon system [73] and G2-based HCV cell culture strains [28]. Whilst these models have allowed considerable advances in the study of HCV replication and development of direct-acting antiviral drugs, they are highly adapted, replicate only in specific cell lines, and the extent to which they predict behaviour of HCV *in vivo* is uncertain. Recent development of G3a and G4a replicons has extended the range of genotypes which can be tested [97, 98], and chimeric variants of JFH-1 have been described [101, 305]. However, these techniques remain impractical for establishing the drug sensitivity of an individual patient's HCV, as defining the drug sensitivity of a single viral isolate requires subcloning the gene for each DAA target and insertion into a subgenomic replicon [306-308]. Furthermore, difficulty in generating replicons with more than one modified locus precludes testing of sensitivity to combinations of DAAs using this technique. For G1 HCV some viral motifs associated with resistance to protease inhibitors have been identified [309] but for other genotypes there is no clear association between viral sequence and drug response [104, 200, 246]. This chapter

presents the work performed to validate the capture-fusion assay as a tool to screen individual patient responsiveness to current and novel antiviral therapies.

5.2 Telaprevir and alisporivir inhibit HCV replication in a genotype-specific manner

We investigated whether replication of patient-derived HCV in the capture-fusion model could be inhibited by antiviral compounds. Telaprevir, a HCV NS3 protease inhibitor, potently inhibits G1 HCV but has little effect against G3 HCV in clinical trials [184, 186, 199]. Conversely, the cyclophilin inhibitor alisporivir is clinically more efficacious against G2 and G3 HCV than G1 and G4 [217]. The ability of the capture-fusion system to accurately reflect these clinical differences in sensitivity was evaluated using serum samples from G1a (N = 9) and G3 (N = 5) infected donors. THP-1 cells infected with the sera were fused with Huh7.5 cells, and the fused cells treated with telaprevir or alisporivir at a range of concentrations for 5 days before quantification of HCV RNA. Dose-response curves were constructed and used to calculate a 50% inhibitory concentration (IC_{50}) for each experiment. Individual patient-derived HCV sensitivity to a wide range of telaprevir/alisporivir doses is shown in Figure 5-1.

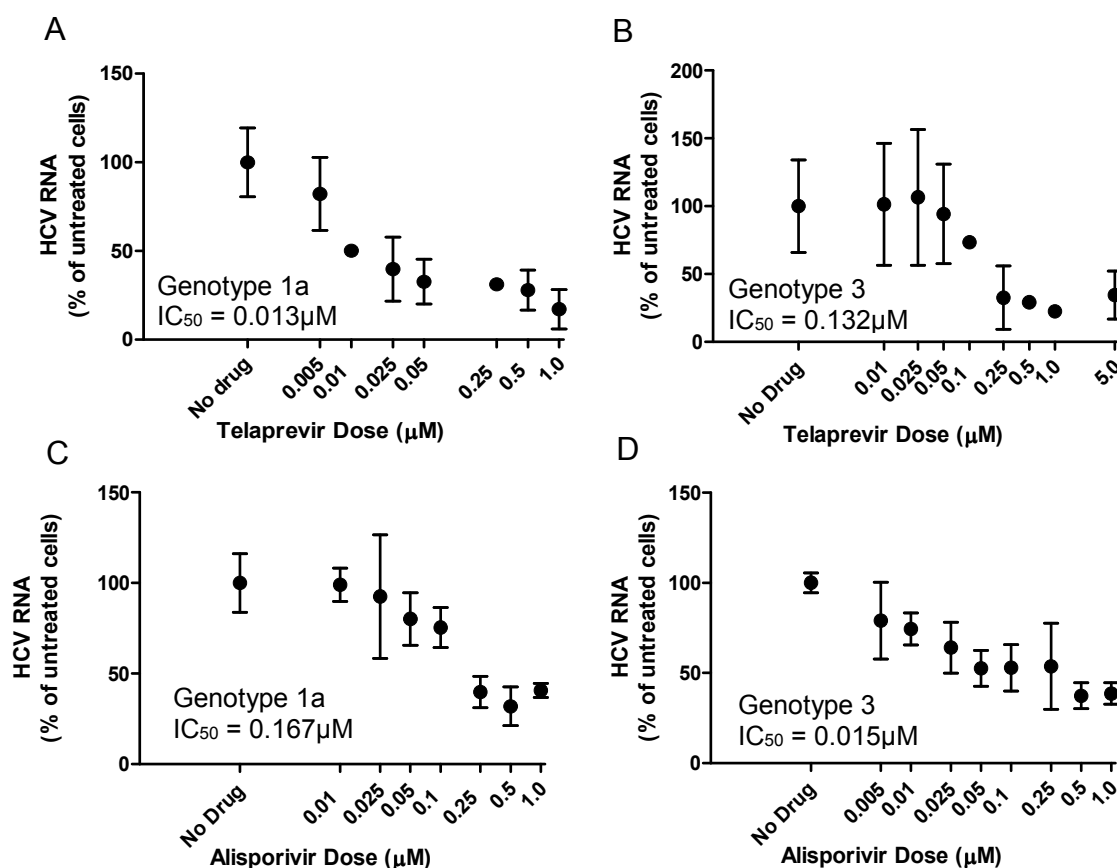


Figure 5-1. Genotype-specific responses to telaprevir or aliporivir treatment in the capture-fusion assay. Each graph shows results of a single capture-fusion experiment using serum from a G1a or G3 HCV infected donor, where the fused cells were treated with a range of doses of telaprevir or aliporivir for 5 days after fusion, prior to quantification of HCV RNA. Graphs show mean \pm s.e.m. of biological quadruplicates.

Based on the sensitivity of diverse viral genotypes to an extensive range of drug concentrations, five drug doses (nil, 0.05, 0.1, 0.5 and 1.0 μM) were selected for use in further experiments. This allowed each capture-fusion drug inhibition experiment to be performed in biological quadruplicates and the resulting HCV RNA measured in a single PCR run. Dose response curves could be constructed for all viral isolates which replicated to quantifiable levels in the assay. Pooled results from 14 donors (9 with G1a HCV and 5 with G3 HCV) are shown in Figure 5-2. Results are normalised to untreated cells to account for variability in replication seen in the capture-fusion assay between samples from different donors. G1 samples were significantly more sensitive to telaprevir than G3

samples (Figure 5-2A, B), but significantly less sensitive to alisporivir (Figure 5-2C, D). Telaprevir and alisporivir IC_{50} s for each sample according to HCV genotype are shown in Figure 5-2E-F.

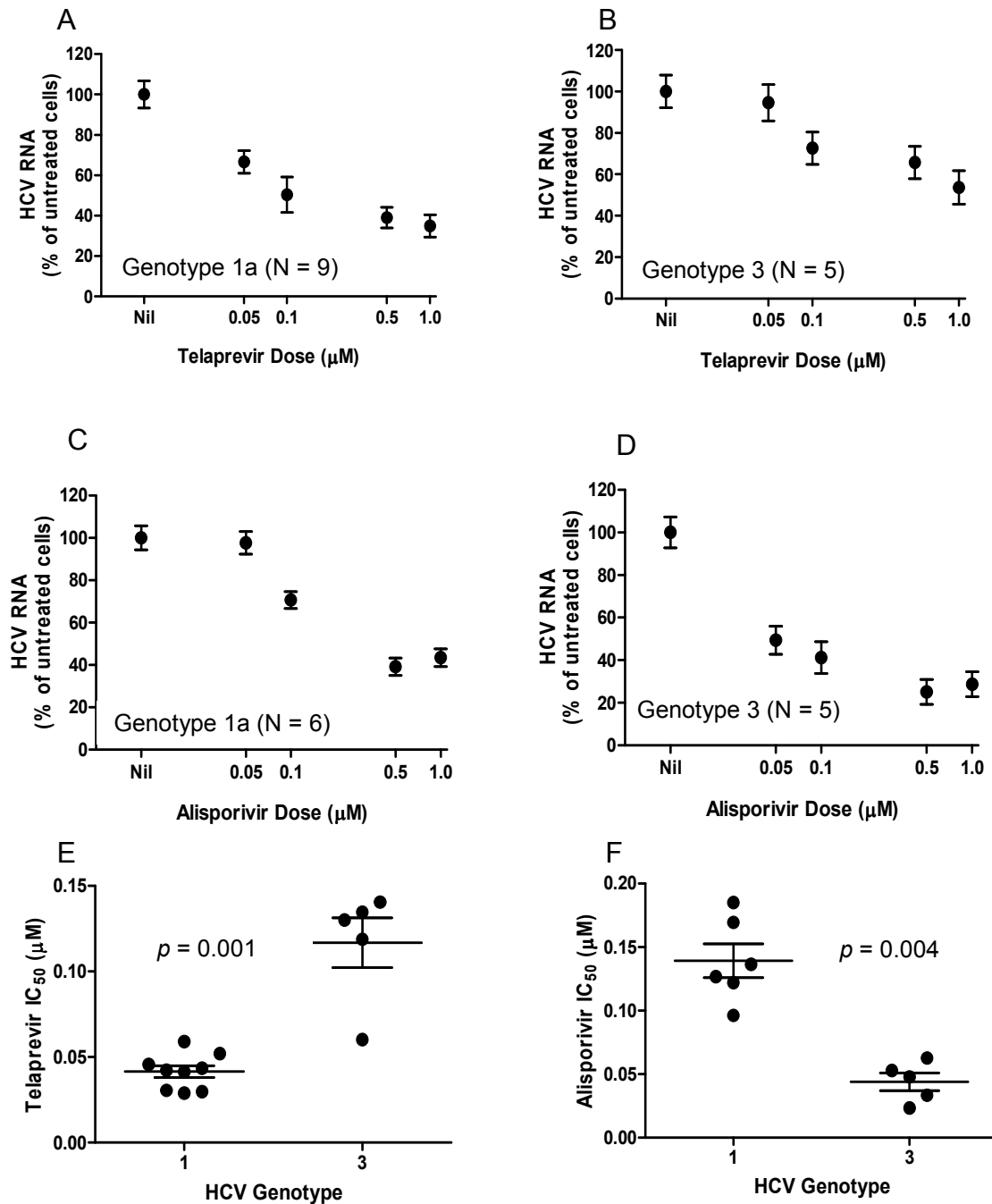


Figure 5-2. Genotype-specific sensitivity of patient-derived HCV to telaprevir or alisporivir in the capture-fusion assay. HCV derived from serum from 9 G1a and 5 G3 donors was treated with varying concentrations of telaprevir or alisporivir in capture-fusion assays, and HCV RNA measured after 5 days. G1a patient-derived HCV was more sensitive to telaprevir than G3 (**A-B**), but less sensitive to alisporivir (**C-D**). Comparison of individual IC₅₀ values showed telaprevir IC₅₀ was 0.042 ± 0.003 for G1 samples versus 0.117 ± 0.015 μM for G3, $p = 0.001$ (**E**). Conversely, alisporivir IC₅₀ was 0.139 ± 0.013 for G1 samples versus 0.044 ± 0.007 μM for G3, $p = 0.004$ (**F**). Graphs show mean \pm s.e.m. P values were calculated using Mann-Whitney U test.

To exclude drug-induced cytotoxicity as a cause of the observed reduction in HCV RNA, cell viability was assessed after exposure to the range of concentrations of telaprevir and alisporivir used in the drug inhibition experiments. Exposure to higher doses of alisporivir caused some loss of viability (up to 25%) but this was insufficient to fully account for inhibition of HCV replication in alisporivir treated samples. Telaprevir exposure did not lead to significant cytotoxicity (Figure 5-3).

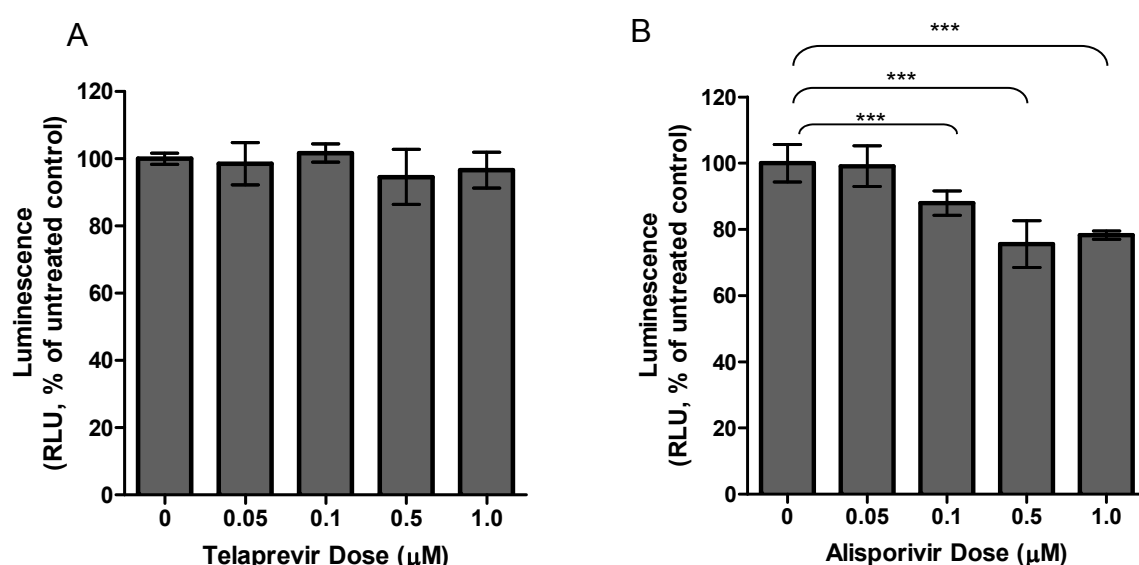


Figure 5-3. Viability of fused cells treated with telaprevir (A) or alisporivir (B). Pre-stimulated THP-1 cells were exposed to HCV infected serum, fused with Huh7.5 cells then cultured for 5 days in the presence of telaprevir or alisporivir at various concentrations. Cell survival was measured using a luminescent cell viability assay and is expressed as a percentage of untreated fused cells. Graphs show mean \pm s.d. of results from single experiments *** $p < 0.001$, calculated using Student's t test.

Initial experiments evaluating the capture-fusion assay as a replication model for all HCV genotypes had suggested that replication of less common viral genotypes could also be inhibited by antiviral drugs, specifically telaprevir, in the assay (see chapter 4). Telaprevir and alisporivir sensitivity of a small number of samples of other viral genotypes was measured and IC_{50} values calculated for each sample (Figure 5-4). G2 isolates were sensitive to both telaprevir and alisporivir, whilst

G4 strains showed a similar alisporivir response to G1, which is in keeping with clinical trial data [217]. Interestingly, some G4 isolates appeared to be poorly sensitive to telaprevir, consistent with the findings of a small clinical trial evaluating telaprevir for G4 HCV [198]. However replication of other G4 isolates was inhibited by telaprevir in the capture-fusion assay, suggesting potential differing susceptibility to telaprevir amongst G4 strains.

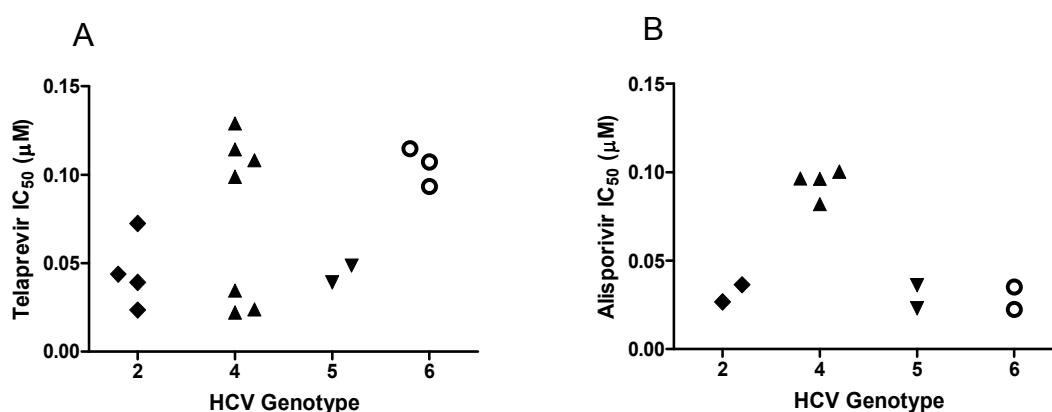


Figure 5-4. Sensitivity to telaprevir and alisporivir can be estimated in patient-derived HCV of all viral genotypes. Graphs show telaprevir (A) or alisporivir (B) sensitivity in individual experiments using serum from patients with genotype 2, 4, 5 or 6 HCV infection.

5.3 Genotypic and phenotypic resistance to direct-acting antivirals can be detected in the capture-fusion assay

Treatment failure in patients receiving telaprevir, pegylated interferon (pegIFN) and ribavirin (RBV) therapy is characterised by an initial response to therapy followed by on-treatment virological breakthrough associated with acquisition of resistance associated variants (RAVs). To explore whether genotypic and clinical telaprevir resistance could be detected as a loss of telaprevir sensitivity in the capture-fusion assay, pre-treatment and post-breakthrough serum samples were obtained from two patients with G1 HCV who initially responded to treatment with

telaprevir, pegIFN and RBV but experienced virological breakthrough by treatment week 12. Sequencing of the first ~200 amino acids of viral NS3 to 1% frequency by pyrosequencing did not identify any known telaprevir resistance associated variants (RAVs) at baseline, but the telaprevir resistance-associated mutations V36M/R155K were found at breakthrough (samples were kindly supplied, with sequencing results, by Dr Tanzina Haque and Mr Malcolm McCartney, Department of Virology, Royal Free Hospital, London). Telaprevir sensitivity of the pre-treatment and post-breakthrough samples for each patient was assessed in the capture-fusion assay and demonstrated a loss of telaprevir sensitivity in the resistant strains (Figure 5-5).

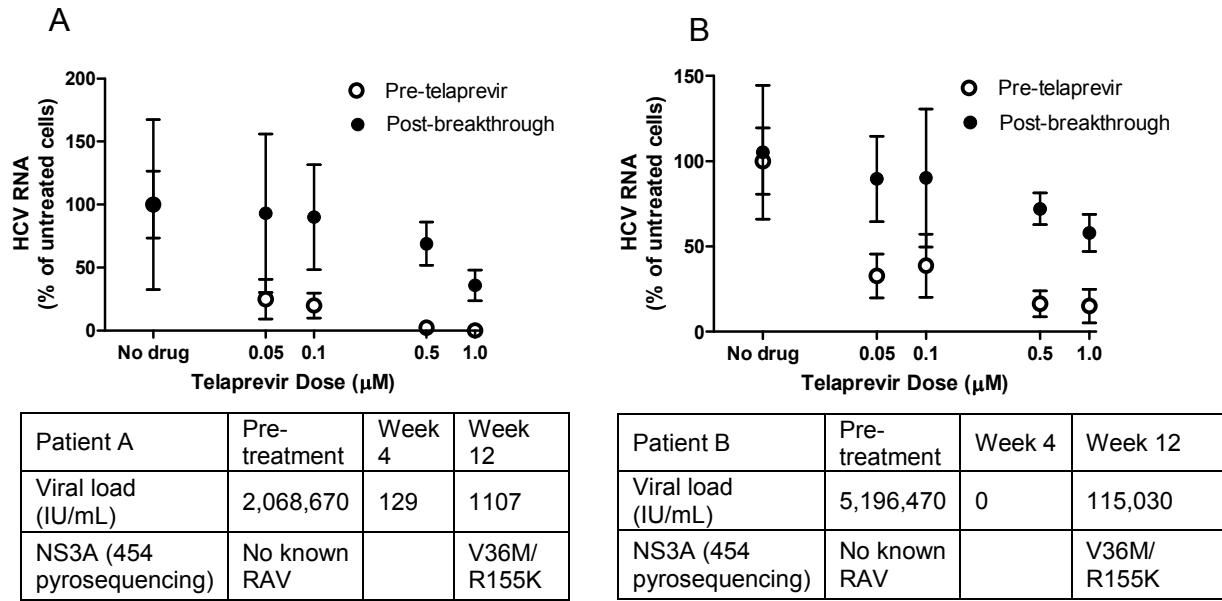


Figure 5-5. Loss of clinical response to telaprevir and acquisition of genotypic resistance correlates with drug sensitivity in the capture-fusion assay. Telaprevir sensitivity of pre-treatment and post-breakthrough sera from 2 patients (**A** and **B**) who initially responded but then broke through treatment with telaprevir, pegIFN and RBV was measured in the capture-fusion assay. Each graph shows mean \pm s.e.m. of a single capture-fusion experiment using pre-treatment serum, and a single capture-fusion experiment using post-breakthrough serum from each patient.

Pre- and post-treatment serum was obtained from one further patient with G1 HCV with a poor clinical response to telaprevir, pegIFN and RBV therapy (circa 1 log₁₀ reduction in viral load at treatment week 4). No telaprevir resistance associated mutations were identified pre-treatment, although V36M/R155K mutations were present at treatment failure (again measured to 1% frequency by 454 pyrosequencing). This strain demonstrated poor telaprevir sensitivity in the capture-fusion assay both before and after telaprevir exposure (Fig. 5-6A). We identified 2 further patients with G1 HCV and a similar poor clinical response to telaprevir, pegIFN and RBV although deep sequencing of the NS3 region of these viral isolates was not available. Pre-treatment sera from these individuals was also poorly sensitive to telaprevir in the capture-fusion assay (Fig. 5-6B, C). This raises the possibility that viral phenotypic sensitivity to telaprevir, as assessed by the capture-fusion assay, may be more predictive of telaprevir treatment response than genotypic sensitivity, at least when assayed using the techniques described.

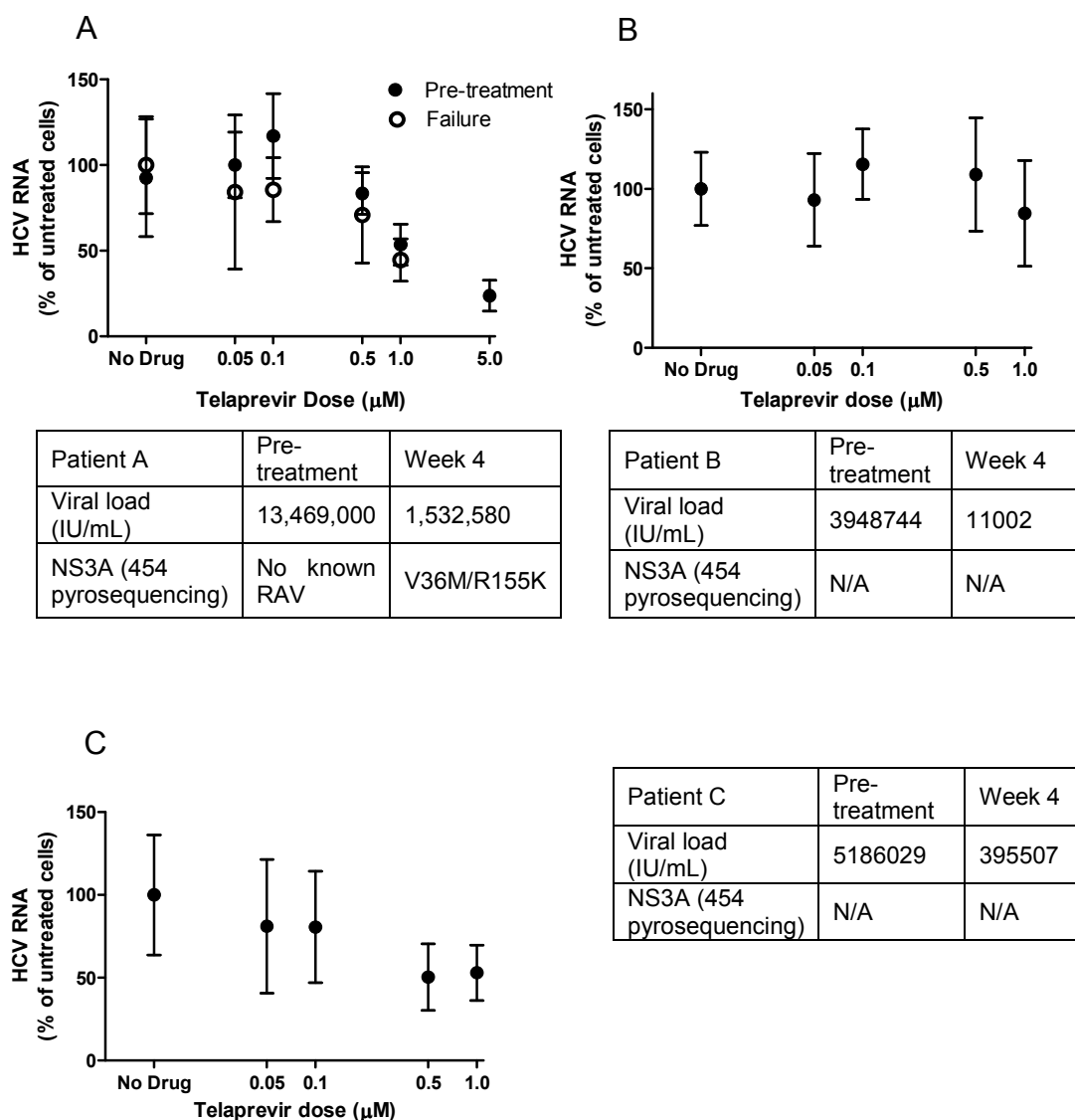


Figure 5-6. Failure to respond to telaprevir-containing treatment correlates with poor pre-treatment telaprevir sensitivity in the capture-fusion assay. Telaprevir sensitivity of pre-treatment and post-failure sera from 1 patient (A) and pre-treatment sera from 2 further patients (B, C) who failed to respond to treatment with telaprevir, pegylated interferon and ribavirin was measured in the capture-fusion assay.

5.4 Interferon sensitivity in the capture-fusion assay correlates with response to interferon in G1 HCV

Interferon sensitivity is thought to be an important determinant of virological breakthrough and acquisition of resistance to novel antiviral drugs, especially in patients who have previously failed to respond to interferon-based therapy. Whether host or viral factors dominate response to interferon treatment is unclear. Archived pre-treatment serum samples from 4 patients with G1 HCV who responded to pegIFN and RBV treatment and 4 patients with a null response to therapy were used to examine whether interferon sensitivity of patient-derived HCV in the capture fusion assay correlated with response to treatment. Pre-treatment HCV from patients who responded to therapy was more sensitive to interferon in the capture-fusion assay than pre-treatment virus from patients with a null response (Figure 5-7).

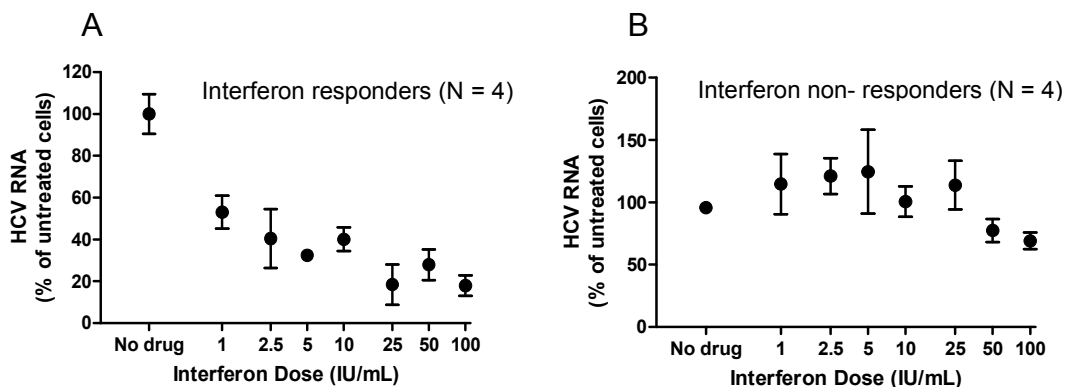


Figure 5-7. Interferon sensitivity in the capture-fusion assay correlates with response to interferon-based therapy. Archived pre-treatment sera from patients who did (**A**, **N = 4**) or did not (**B**, **N = 4**) respond to pegIFN and RBV were used in capture-fusion experiments. Fused cells were treated with interferon α -2a at a range of doses for 5 days prior to HCV quantification. Graphs show mean \pm s.e.m. of pooled results from all 4 experiments in each group.

5.5 Ribavirin sensitivity in the capture-fusion assay correlates with relapse after interferon and ribavirin therapy in G3 HCV

In contrast to G1, the predominant mode of treatment failure in patients with G3 HCV infection is relapse. Relapse rates are higher in patients who receive suboptimal ribavirin dosing and some investigational treatment regimens without ribavirin [310, 311]. Archived pre-treatment sera from 10 cirrhotic G3 patients treated with pegIFN and RBV, 4 with sustained virological response (SVR) and 6 who relapsed, were used to investigate whether pre-treatment interferon or ribavirin sensitivity in the capture-fusion assay correlated with treatment outcome. No difference in interferon sensitivity was seen, but pre-treatment HCV from patients with SVR was more sensitive to ribavirin in the assay than virus from patients who relapsed (Fig. 5-8).

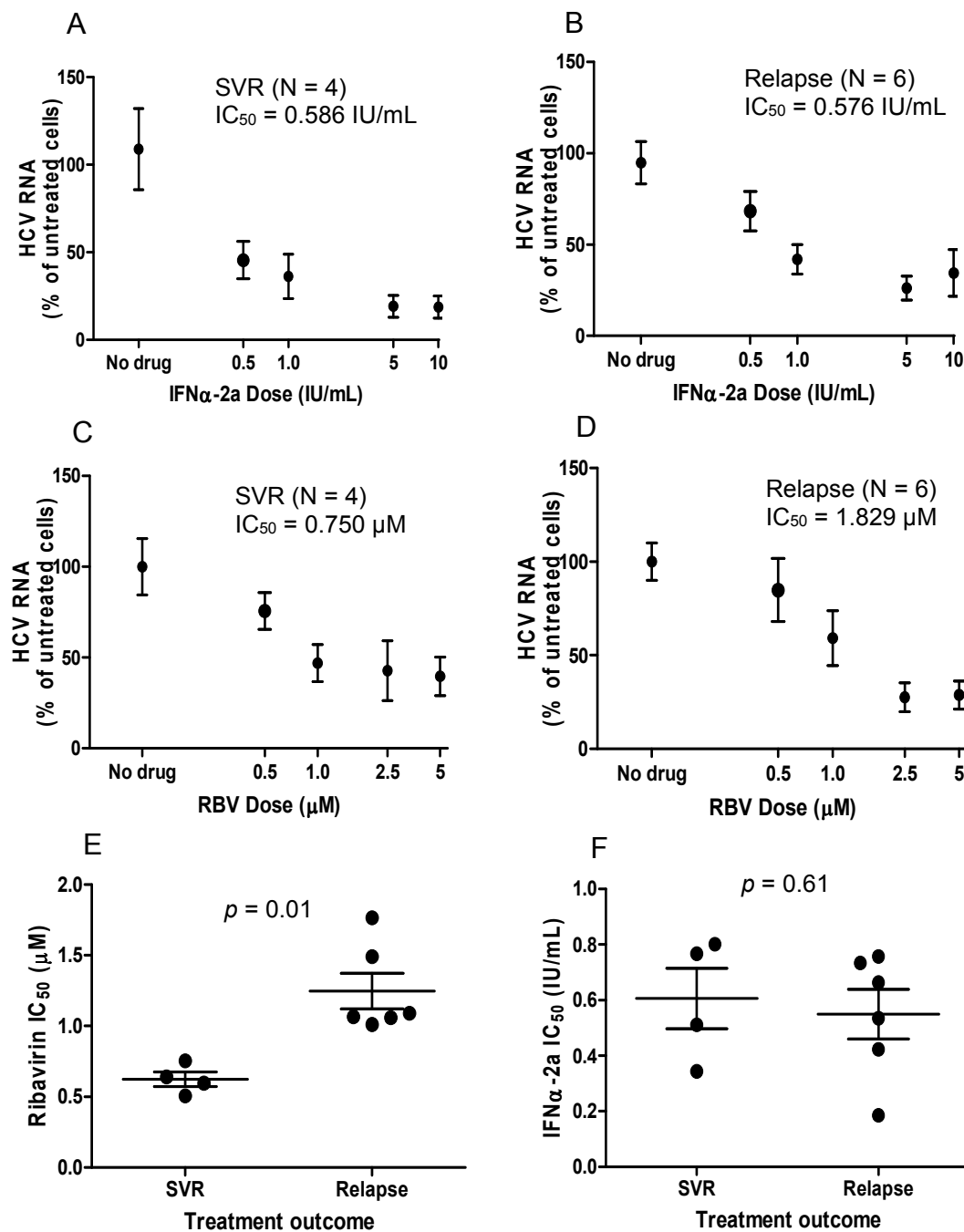


Figure 5-8. Pre-treatment ribavirin sensitivity in the capture-fusion assay correlates with treatment response in cirrhotic G3 patients treated with interferon/ribavirin. Using archived pre-treatment sera from 10 cirrhotic G3 patients who relapsed (N = 6) or achieved SVR (N = 4) following pegIFN and RBV treatment, interferon (A, B) and ribavirin (C, D) sensitivity was measured in the capture-fusion assay. Pre-treatment sera from patients with SVR were significantly more sensitive to ribavirin, but not interferon, than sera from relapsers (E, F). Graphs A-D show mean \pm s.e.m. of pooled results from all experiments in each group. Graphs E and F show mean \pm s.e.m. of IC_{50} values calculated from each experiment. P values were calculated using Mann-Whitney U test.

5.6 Sensitivity to telaprevir in the capture-fusion assay correlates with clinical response

Although telaprevir is poorly effective in G3 HCV, a subgroup of patients (~30%) do show some antiviral response [199]. To establish if the capture-fusion assay could be used to distinguish patients with telaprevir-sensitive G3 HCV, pre-treatment serum samples were obtained (blinded to virological response) from eight patients with G3 HCV who had been treated with telaprevir monotherapy in a clinical trial. Using the capture-fusion assay, telaprevir sensitivity of each sample was determined. Telaprevir sensitivity measured in the capture-fusion assay correlated well with clinical response. In contrast, a biochemical phenotyping assay, in which the viral NS3 was subcloned and enzymatic activity assessed in the presence and absence of telaprevir using fluorescence resonance energy transfer cleavage, correlated poorly with clinical outcome [104] (samples and results of the biochemical assay kindly supplied by Gaston Picchio, Janssen Virology) (Figure 5-9). Three samples with the greatest telaprevir sensitivity in the capture-fusion assay corresponded to the three patients who had shown a clinical response to telaprevir monotherapy before acquiring resistant viral strains. These results indicate that the capture-fusion assay may be useful to predict individual patient response to telaprevir, and to distinguish patients with G3 infection who may respond to telaprevir therapy from those who will not.

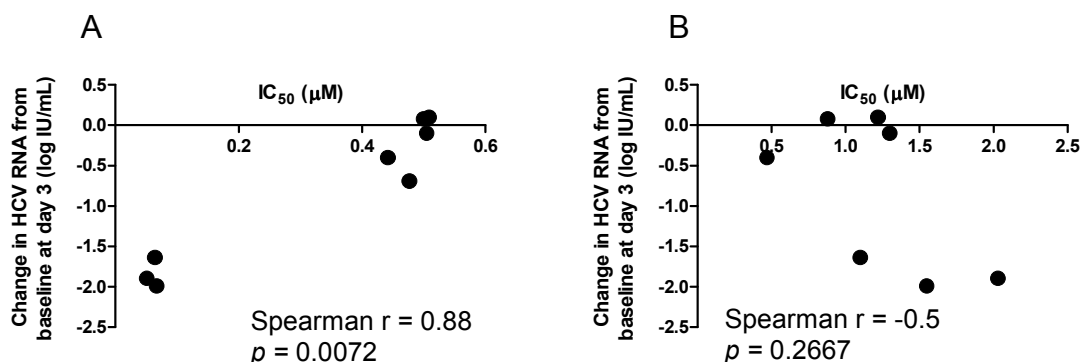


Figure 5-9. Correlation between pre-treatment telaprevir sensitivity and response to telaprevir in 8 patients with G3 HCV. Clinical response to telaprevir monotherapy is shown on the y-axes, and could be predicted by the capture-fusion assay (**A**), but not by a biochemical phenotyping assay (**B**). Graphs show IC₅₀ values from individual experiments using serum from 8 G3 HCV infected donors prior to telaprevir treatment. P values were calculated using Spearman rank correlation test.

5.7 Use of the capture-fusion assay to investigate anti-HCV activity of a novel antiviral drug

To further explore the range of antiviral drugs for which patient-derived HCV sensitivity could be investigated using the assay, the novel antiviral agent SB 9200 was supplied by SpringBank Pharmaceuticals.

SB 9200 is a host-targeting antiviral compound which, at the time of writing, is in Phase 1 clinical trials for treatment of chronic HCV infection. Its precise mechanism of action is not fully elucidated but it is thought to involve activation of components of the innate antiviral immune response, including the double stranded RNA helicase RIG-I and the pattern recognition receptor NOD-2. It is a broad spectrum antiviral, with activity demonstrated against HCV, HBV, respiratory syncytial virus and norovirus [312].

The dose range of SB 9200 used in capture-fusion experiments was established by treating HCV replicon cells with various concentrations of SB 9200 for 5 days, before quantification of HCV RNA and construction of a dose-response curve. Inhibition of the HCV replicon was demonstrated over the range 0-10 μ M SB 9200, and so the doses selected for use in the capture-fusion assay were 0, 0.01, 0.1, 1.0 and 10 μ M.

To demonstrate that SB 9200 was effective against diverse HCV genotypes in the capture-fusion assay, serum samples from donors infected with G1 (N = 6), G2 (N = 2), G3 (N = 7), G4 (N = 3) and G6 (N = 2) HCV were used in capture-fusion experiments. Fused cells were treated with SB 9200 for 5 days before quantification of HCV RNA. Isolates of all viral genotypes tested were sensitive to SB 9200 in the capture-fusion assay (Fig 5-10), although G3 isolates were significantly more sensitive to SB 9200 than G1 samples. Single samples were available from a G1 patient who did not respond to telaprevir (telaprevir sensitivity in the capture-fusion assay shown in Fig 5-6C) and from a G3 patient who had repeatedly relapsed after pegIFN and RBV therapy. Both of these samples were sensitive to SB 9200 in the capture-fusion assay (Fig. 5-10 F, G).

Together, these results suggest that SB 9200 may be a useful host-targeting anti-HCV agent with pan-genotypic antiviral activity and may be effective against viral strains which are poorly responsive to other antiviral agents. The capture-fusion assay may be a useful tool to identify genotypic range and efficacy of novel anti-HCV drugs at the early stages of clinical development.

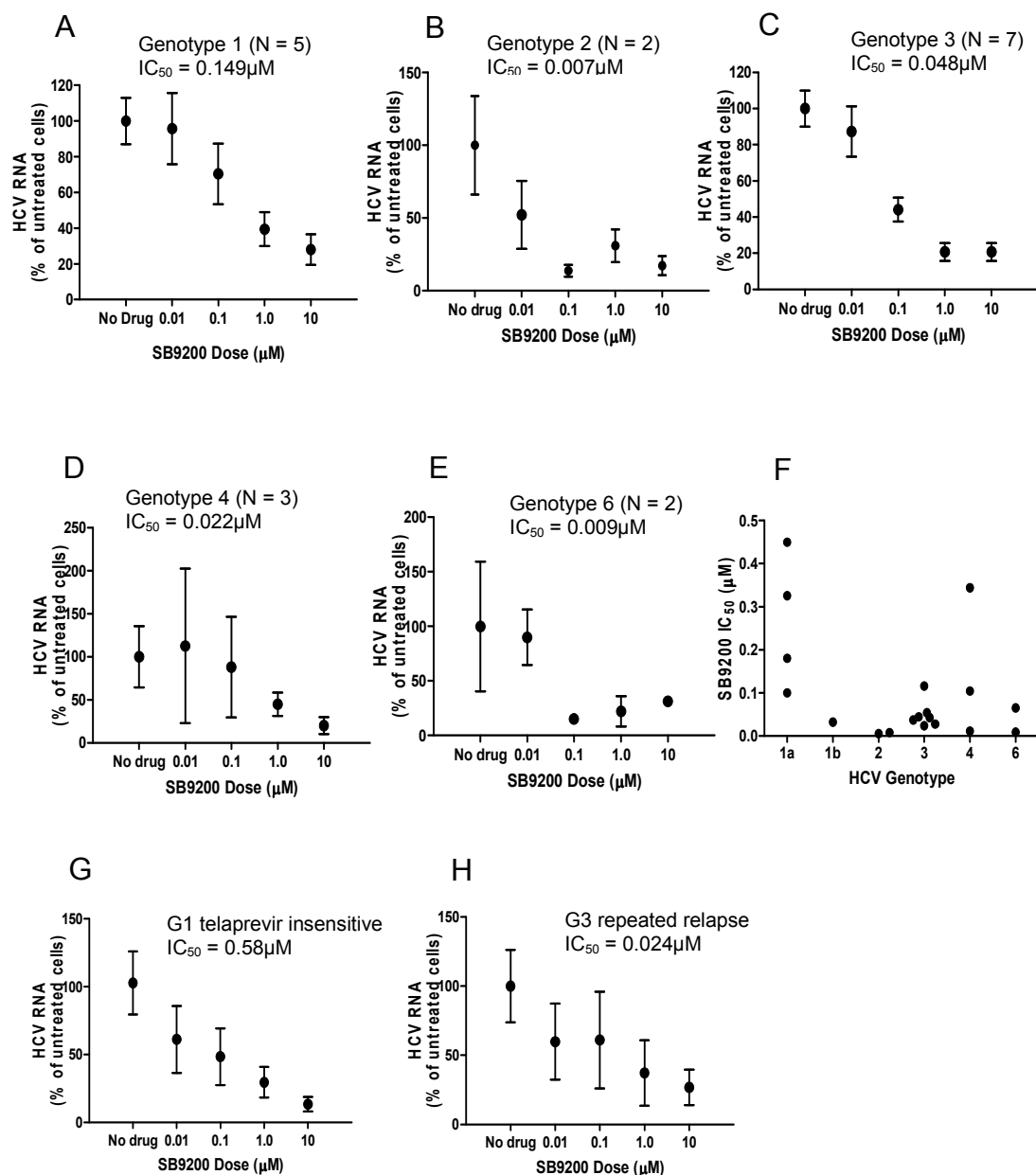


Figure 5-10. Sensitivity of patient-derived HCV to SB 9200 in the capture-fusion assay. Serum samples from donors chronically infected with HCV G1 **A**, N = 5); G2 **B**, N = 2); G3 **C**, N = 7); G4 **D**, N = 3) or G6 **E**, N = 2) were used in separate capture-fusion experiments. After fusion, cells were treated with a range of concentrations of SB 9200 for 5 days before quantification of HCV RNA and construction of dose-response curves. SB 9200 IC_{50} for each sample is shown in **F**. In addition, the capture-fusion assay was used to measure SB 9200 sensitivity of HCV from a patient with G1 HCV who had failed to respond to telaprevir, pegIFN and RBV **G**) and from a patient with G3 HCV who had relapsed on 3 occasions after pegIFN and RBV therapy **H**). Graphs show mean \pm s.e.m.

5.8 Discussion

The work presented in this chapter demonstrates that replication of HCV in the capture-fusion assay can be inhibited by antiviral drugs in a manner that reflects both genotype-specific and patient-specific drug response. This assay represents the first replication system for HCV where drug responsiveness of individual patient-derived HCV can be tested *in vitro*, and where drug efficacy can readily be tested in genotypes other than G1 (replicon) or G2 (cell culture HCV strains).

50% inhibitory concentrations (IC_{50}) for a given drug often vary in published literature, which may be due to experimental differences such as the assay used (e.g. cell culture based or enzymatic), the time of exposure to the drug or the range of drug concentrations employed. IC_{50} values obtained in telaprevir or alisporivir inhibition experiments using serum from G1 donors in the capture fusion assay are comparable to IC_{50} values obtained in the replicon system (G1b) [313, 314], further supporting the validity of the capture-fusion assay as tool to screen drug sensitivity of patient-derived HCV.

A very limited number of genotype 4, 5 and 6 isolates were available for testing in the assay, as these genotypes (especially G5 and G6) are rare in the UK. Very little clinical data is available for these viral genotypes and there is no published data on clinical efficacy of telaprevir in G5 or G6 HCV. A small clinical trial suggested that G4 HCV is poorly sensitive to telaprevir [315], but divergent responses to protease inhibitors have been observed amongst isolates of the same genotype [199, 246], which could not reliably be predicted by presence of resistance-associated motifs [104, 200]. The results obtained in the capture-

fusion assay suggest that there may be strain-specific differences in telaprevir sensitivity amongst G4 isolates, whilst at least some G5 isolates may respond well and some G6 isolates may respond poorly to telaprevir. The results of our blinded study where three of eight G3 samples which showed a clinical response to telaprevir were accurately identified in the capture-fusion assay suggests that this assay may represent a valuable screening tool to identify which non-G1 infected patients may benefit from protease inhibitor therapy. However, correlating viral sensitivity in the capture-fusion assay with sustained clinical response to therapy will require further work.

Notably, we identified a single patient with a poor therapeutic response to telaprevir whose virus was phenotypically resistant to telaprevir in the absence of any detectable telaprevir resistance associated variant (RAV) by deep sequencing of the viral NS3 region. Two further patients were subsequently identified who also failed to respond to telaprevir therapy with phenotypically resistant virus pre-treatment, although viral NS3 sequencing was not available for these samples. It is possible that a pre-existing RAV was present at <1% frequency and so was not detected by 454 sequencing, and was subsequently selected during treatment. Alternatively, these results raise the possibility of novel resistant variants to telaprevir which may exist in the population and may lie outside the classical resistance associated regions. Further work will be required to delineate this region and large-scale studies will be needed to identify the frequency with which these variants occur.

The predictive value of pre-existing resistance associated variants (RAVs) on outcome of telaprevir treatment is unclear. Pre-existing RAVs conferring low-level telaprevir resistance have been identified by population sequencing in up to 3% of treatment-naïve or pegIFN/ribavirin treatment-experienced individuals, but their presence does not appear to influence the outcome of subsequent telaprevir-containing treatment, except in the context of a poor interferon response [259, 262]. In clinical trials the number of participants with primary failure of telaprevir-containing therapy (as distinct from virological breakthrough) has not been reported [184, 186]. However, in a large series of cirrhotic, treatment-experienced G1 patients treated with telaprevir, pegIFN and RBV, 10% failed to respond to triple therapy [251]. Therefore, primary non-response to telaprevir-based therapy may become a significant issue as protease inhibitor use becomes more widespread, particularly in patients who have previously failed treatment with pegIFN and ribavirin. A phenotyping assay such as the capture-fusion assay may be of value to identify these patients pre-treatment.

SB 9200, a novel antiviral in early phase clinical trials for treatment of HCV, is thought to exert its antiviral effects primarily through activation of RIG-I signalling, although NOD2 has also been implicated [312]. The fact that clear inhibition of HCV replication by SB 9200 was seen in the fusion model was interesting, given that the RIG-I pathway is defective in Huh7.5 cells [285]. This implies that the role of NOD2 activation in the mechanism of action of SB 9200 is greater than previously appreciated, or that it activates part of the RIG-I signalling pathway downstream of the defect present in Huh7.5 cells.

The clinical response to pegIFN and ribavirin therapy for HCV is influenced both by host characteristics, such as IL28B genotype [165, 166] and baseline interferon stimutable gene (ISG) expression [179, 181], and viral genotype. Overall, with the exception of the interferon sensitivity determining region (ISDR) in the HCV NS5A, where an accumulation of mutations is associated with higher rates of SVR in G1b HCV [163], host factors are thought to dominate interferon responsiveness. In an assessment of viral interferon sensitivity in the capture-fusion assay in eight patients with extreme responses to therapy, we found reduced interferon sensitivity in patients who showed no clinical response to treatment. The archival samples analysed could not be correlated with IL28B genotype and insufficient samples were available to assess ribavirin sensitivity. Although the number of samples analysed was very small, these findings suggest viral characteristics may play a role in determining outcome of interferon-based therapy. Further studies will be needed to investigate the complex interactions between host characteristics, especially IL28B genotype, viral interferon sensitivity and clinical outcome.

The mechanisms by which ribavirin exerts its antiviral effects in treatment of chronic HCV remain uncertain, but it remains an important component of treatment, even in the emerging era of interferon-free treatment regimens [316]. Amongst patients with G3 HCV cirrhosis, we noted a reduced pre-treatment response to ribavirin in patients who relapsed when compared to patients who achieved SVR and these data suggest that ribavirin sensitivity may be an important determinant of response in this genotype. Whether this finding extends to non-cirrhotic patients, relapse in patients infected with other genotypes, or

relapse following treatment with ribavirin in combination with drugs other than interferon, will require further investigation.

6.0 Discussion and Further Work

This project aimed to investigate factors that influence response to antiviral treatment amongst patients with chronic HCV infection. In this work, we have modified a fusion assay (previously developed in the group) to use cell culture monocytes to “capture” HCV from sera of patients infected with diverse viral strains. In the majority of samples viral replication is seen after fusion of HCV-exposed monocytes with hepatoma cells, and can be inhibited by antiviral drugs. We have undertaken work to validate this “capture-fusion” approach as an assay to screen patient sensitivity to antiviral drugs, and to investigate mechanisms underlying HCV infection of monocytes and the role this may play in relapse after antiviral therapy. We have demonstrated that drug sensitivity in the capture-fusion assay reflects genotypic patterns of treatment response, as well as genotypic and clinical drug resistance. Furthermore, our work suggests that HCV may utilise CD64 to enter monocytes, and both pre-treatment ribavirin sensitivity and persistent HCV harboured in monocytes at the end of interferon and ribavirin therapy may be implicated in relapse.

The most significant limitations of the capture-fusion approach are the low level of viral replication achieved, and the short period for which replication can be sustained (up to seven days) as the fused cells do not survive passage. HCV RNA could only be detected and quantified using a sensitive PCR assay. Other methods to detect viral replication (detection of protein production by indirect immunofluorescence or western blot, and alternative replication detection systems, such as the SEAP reporter cell line J20) were unsuccessful, most likely due to insufficient sensitivity. Negative strand PCR, widely used by others to

confirm HCV replication, was not attempted as its sensitivity is 10-1000-fold lower than that of positive strand PCR, and so would have been unlikely to have yielded positive results. Low level replication precluded detailed study of the mechanisms underlying replication in the hybrid cells, which in turn made it difficult to develop strategies to enhance replication. Empirical use of techniques found to enhance viral replication of the HCV cell culture strain JFH-1 met with varying success.

To demonstrate HCV replication, monocyte fusion experiments and capture-fusion experiments relied on accumulation of HCV RNA over time, and dose-dependent reduction in HCV RNA following treatment with antiviral drugs. Although the inability to demonstrate HCV replication with any other technique than PCR is a significant limitation of this work, the PCR assay used was demonstrated to be highly sensitive, with a lower limit of detection of 10 copies and low intra- and inter-assay variation. Over the course of this project, many monocyte fusion and capture-fusion experiments were performed using samples from a large number of patients, infected with diverse HCV genotypes. The extent to which HCV RNA increased over time after fusion was highly variable between samples and in some cases little increment was seen, implying viral turnover or very low level replication only. However, the vast majority of fusion experiments yielded quantifiable HCV RNA 5 days after fusion, and the results were remarkably consistent when experiments were repeated using the same sample. Regardless of the level of HCV RNA accumulation seen after fusion, viral RNA levels were consistently reduced in a dose-dependent manner by exposure to antiviral drugs, and this correlated with known genotypic and clinical drug sensitivity. Together, these findings strongly suggest that low level viral

replication occurs in the capture-fusion system. The level of replication seen in the current assay is sufficient to allow testing of drug response in most samples, but is a significant limitation to the use of the assay to study replication of patient-derived HCV in the hybrid cells.

Use of digital PCR may allow more precise quantification of HCV RNA in the capture-fusion model. This novel technique relies on distribution of the sample nucleic acid into thousands of individual PCR reactions. Each reaction will receive a target molecule, yielding a positive result, or no target, yielding a negative result. The fraction of negative reactions is used to generate absolute quantification of target nucleic acid in the sample, without the need for a reference standard. A Poisson distribution model corrects for the possibility that some reactions receive more than 1 input copy of target nucleic acid. Target detection occurs with increased sensitivity and specificity compared to real time PCR, as partitioning of samples reduces competition and background DNA. The increased precision of quantification of low copy number target offered by digital PCR would enhance the reliability of HCV RNA detection in the capture-fusion assay. This may represent a useful option for future studies, as the technology becomes more widely available.

6.1 The capture-fusion assay as a tool to study drug sensitivity of patient-derived HCV

The recent advances in *in vitro* model systems to study HCV replication have permitted enormous progress in understanding of the HCV life cycle and development of novel direct-acting antiviral therapies (DAAs). However a system

permitting the study of patient-derived HCV is still lacking. Patient-derived HCV does not infect hepatoma cells in culture, and the reasons for this remain poorly understood. The replicon and HCVcc systems (JFH-1) are based on highly adapted viruses replicating within an immortalised liver cell line [28, 73, 99]. Infection of primary monocyte/macrophages and T cells by patient-derived viral strains has been described [119, 124, 126], as well as chronic infection of transformed B and T cell lines *in vitro* using patient-derived virions [128, 129, 317]. Hepatocyte-like cells derived from human embryonic stem cells or induced pluripotent stem cells support JFH-1 replication, but their use as a replication system for patient-derived HCV has not yet been reported [318, 319]. Attempts to use primary human hepatocytes (PHH) have been hampered by poor proliferation, limited viability in culture and restricted availability of cells [107].

In this work, we have developed a model in which patient-derived HCV of all viral genotypes will replicate. For the majority of viral isolates, replication occurs at a sufficient level and for a sufficient duration to permit testing of sensitivity to antiviral drugs. It is possible that minor viral strains are selectively associated with monocytes, or that monocyte-associated virus has undergone adaptive mutations which render it un-representative of the patient's viral quasispecies. Due to the low level of viral replication obtained, we were unable to perform extensive sequencing studies to investigate this. However the consistency between drug sensitivity seen in the assay, known genotype-specific responses to antiviral drugs and individual patient responses to drug therapy, implies that any viral selection or adaptive mutations are not significant for purposes of determining drug sensitivity.

The clinical response to pegIFN/RBV therapy for patients with HCV is dominated by viral genotype, host single nucleotide polymorphisms (SNPs) upstream of the *IL28B* gene [165, 166], and baseline host expression of interferon stimable genes (ISG) [178, 179]. In an assessment of pre-treatment viral interferon sensitivity in eight patients with diverse responses to pegIFN/RBV therapy, we found reduced viral interferon sensitivity in patients who showed no clinical response. The archival samples analysed could not be correlated with *IL28B* genotype and insufficient samples were available to assess ribavirin sensitivity or ISG expression. Further studies will be needed to investigate the complex interactions between host *IL28B* genotype, interferon responsiveness, viral interferon sensitivity and clinical outcome.

We have demonstrated that sensitivity of patient-derived HCV can be determined to several antiviral agents with widely differing mechanisms of action in the capture-fusion system. Antiviral therapy for HCV is rapidly evolving to include multi-drug combinations of DAAs. Although in clinical trials cure rates with these combination therapies are high [228, 240, 247, 320], rates of treatment failure in real-world settings are likely to be greater. To avoid development of 'multi-drug resistant' HCV strains, careful selection of 'second line' regimens will be required. Identification of the most effective treatment regimens in this scenario would be facilitated by phenotyping assays. Combinations of DAAs have not been tested as yet in the capture-fusion assay, and establishing its ability to predict treatment outcome to combination therapy is an important area for further work.

For the protease inhibitor telaprevir, our assay accurately identified viruses with known resistant variants and, of interest, we identified a single patient with wild type protease sequence and a poor therapeutic response to telaprevir whose virus was phenotypically resistant to telaprevir. These data suggest that novel resistant variants to telaprevir may exist and lie outside the classical resistance associated regions. Further work will be required to delineate this region and identify the frequency of these variants. In a blinded study using the capture-fusion assay, we distinguished three G3 samples which had shown some response to telaprevir monotherapy in a clinical trial, from five samples with no telaprevir response [199]. These three samples could not be distinguished by genotyping, or by a commercial biochemical phenotyping assay [104]. Together, these findings suggest there may be patient-specific variability in response to DAA-based therapy which cannot be predicted by current genotyping or phenotyping assays. Future optimal treatment regimens may need to be patient-specific, especially in patients who have already failed therapy, emphasising the need for a reliable phenotyping assay.

6.2 Relapse after antiviral therapy

We explored the possibility that monocytes play a role in relapse after anti-viral treatment by acting as a sanctuary site for HCV during therapy. An association between HCV detectable in monocytes at the end of therapy and relapse has been reported in patients co-infected with HIV and HCV [146], although patients co-infected with HIV may demonstrate increased replication of HCV in monocytes/macrophages compared to HCV mono-infected patients [271].

In patients with genotype (G)3 HCV, the viral genotype most prone to relapse following pegylated interferon and ribavirin (pegIFN/RBV) therapy, we found an association between viable HCV RNA in end-of-treatment monocytes and relapse after treatment. However, when we studied end-of-treatment monocytes from G1 HCV-infected patients treated with the HCV nucleoside polymerase inhibitor sofosbuvir and ribavirin, we found no evidence of viable HCV RNA in any sample, regardless of treatment outcome. Whether this reflects genotypic differences in treatment response or therapeutic differences in targeting of extrahepatic virions remains to be investigated.

Using the capture-fusion model, we have also demonstrated that impaired viral ribavirin sensitivity pre-treatment was associated with relapse following pegIFN/RBV therapy in a cohort of cirrhotic patients with G3 HCV. One possible explanation for these findings is that ribavirin plays a critical role in treatment of extrahepatic HCV. Poorly ribavirin sensitive virus may be sequestered in extrahepatic sites (such as monocytes) during treatment, where it could conceivably be relatively protected from the effects of exogenous interferon if the level of turnover or viral replication is low. With regards to sofosbuvir/ribavirin therapy, the lack of apparent viable HCV RNA associated with end-of-treatment monocytes could be attributable to acquisition of adaptive mutations conferring reduced susceptibility to sofosbuvir in the poorly ribavirin-sensitive virions sequestered in extrahepatic sites during therapy. These resistance-associated variants (RAVs) often have compromised replicative fitness [207], which may explain why replication was not seen after fusion of end-of-treatment monocytes with Huh7.5 cells. Further investigation will be required to explore this hypothesis,

including sequencing of viral strains from pre-and post-treatment serum and monocytes and phenotyping in chimeric replicons.

6.3 HCV infection of monocytes

Further work employing the capture-fusion assay would be greatly assisted by enhancement of viral replication in the assay. Strategies reported by others to boost HCV replication in Huh7.5 cells, including treatment with the sonic hedgehog pathway agonist SAG and the cytokine osteopontin [292, 293], had limited and variable effect in the capture-fusion model. It is likely that successful and robust enhancement of replication in this assay will require greater understanding of the mechanisms permitting viral replication after fusion of infected monocytes with hepatoma cells. In common with others, we were unable to infect THP-1 cells with cell culture HCV (HCVcc; JFH-1) [122]. More recently, THP-1 have been successfully transfected with modified JFH-1 RNA [321]. This technique may assist in establishing a greater level of replication in the capture-fusion assay, and help break the cycle of low level replication impeding investigation of mechanisms underlying viral replication in the fusion model, which in turn limits development of strategies to boost replication. Further understanding of extrahepatic HCV entry and replication may ultimately assist in development of treatment strategies to prevent relapse after antiviral therapy.

Our work has suggested a role for Fcγ receptor I (CD64) in attachment of patient-derived HCV RNA to THP-1, which then replicates after fusion with Huh7.5. However, CD64 blocking did not completely abrogate HCV replication after fusion, and so this is unlikely to be the sole means of uptake. Although we

demonstrated that a proportion of HCV RNA is internalised into THP-1 cells after incubation with patient serum, further work is needed to establish whether viral internalisation is required for replication after fusion. One hypothesis is that HCV is protected from degradation after uptake into monocytes by specific mechanisms, perhaps by segregation within intracellular compartments. This virus may then replicate after transfer to an environment rich in permissive factors for HCV replication. In support of this hypothesis, others have demonstrated that HCV virus-like particles were protected from lysosomal degradation after DC-SIGN mediated uptake into dendritic cells or L-SIGN-mediated uptake into THP-1 cells [299]. Targeting to a non-lysosomal compartment appeared to be ligand-dependent, so whether this pathway is relevant to uptake of patient-derived virions into THP-1 cells remains to be established. A series of experiments involving L-SIGN blocking and/or knockdown, fusion after trypsin treatment of HCV-exposed THP-1 and use of recently described fluorescent constructs which allow intracellular localisation of HCV replication [295] may help test this hypothesis.

Alternatively, it is possible that internalisation of HCV into monocytes is not required for replication after fusion with Huh7.5. The fusion process may bypass any blocks in entry of patient-derived HCV into Huh7.5 cells, and “loading” of monocytes with HCV may result in bulk delivery of virus into the cell which overwhelms innate antiviral immune responses, permitting low level viral replication to occur. In support of this hypothesis, cytoplasmic sensing of dsRNA by retinoic acid inducible gene-I (RIG-I) is defective in Huh7.5, permitting enhanced HCV replication relative to the parent cell line (Huh7) in which RIG-I

function is intact [285]. Furthermore, we have demonstrated that the process of cell fusion of HCV-infected monocytes with Huh7.5 cells does not trigger expression of interferon stimutable genes, although their expression remains inducible by exogenous interferon.

Whether transfer of internalised or surface-bound HCV into Huh7.5 is more important for replication after fusion, it appears that viral replication is facilitated by transfer to a more permissive environment. Cell fusion studies have previously been used to exclude dominant restriction factors as the limitation on HCV replication in non-liver cells [284]. A number of host factors are required for HCV replication which are present in Huh7.5 cells, including phosphatidylinositol 4-kinase type III- α (PI4KIII- α) [63, 64] and the liver-specific micro-RNA miR-122 [55]. The importance of these factors in HCV replication after fusion could be explored by knockdown in Huh7.5 cells before fusion. Ectopic expression in THP-1 cells of critical permissive factors for replication after fusion might permit replication of patient-derived HCV in these cells, dispensing with the need for cell fusion and potentially enhancing the viability of the system for high-throughput screening of patient-derived HCV sensitivity to antiviral therapies.

6.4 Extrahepatic HCV replication

Extrahepatic replication of HCV has long been controversial. Over recent years, particularly with advances in technology with increased sensitivity to detect very low amounts of viral RNA and non-structural proteins, a consensus view has emerged that HCV probably does turn over or replicate at very low levels in extrahepatic sites, including PBMCs [112-116]. It has been suggested that such

low level replication permits the virus to evade intracellular innate antiviral detection mechanisms [139]. The work presented in this thesis lends support to this hypothesis by using an entirely novel technique to detect the presence of viable HCV RNA in patient-derived monocytes. We were unable to demonstrate significant HCV replication within monocytes or THP-1 cells, but the demonstration of replication after fusion with Huh7.5 cells strongly supports the presence of viable HCV RNA associated with these cells.

The presence of viable HCV in patient-derived monocytes has potential significance in several aspects of HCV infection, including establishment of chronic HCV infection, reinfection of the graft after orthotopic liver transplantation and relapse after antiviral therapy. HCV has been successfully transmitted to a chimpanzee by transfusion of PBMC from an infected individual [322] and to immunodeficient mice by transfusion of human PMBCs from an HCV-infected donor [323]. Following orthotopic liver transplantation, reinfection of the graft is universal amongst HCV-infected recipients. The primary source of reinfection appears to be serum-derived virus but reinfection from PBMCs has been implicated [144] and graft infection is also described amongst recipients who are serum HCV RNA negative [145]. The source of infection in these patients has not been clearly identified, but reinfection by HCV sequestered in monocytes/macrophages or other PBMCs is a possibility and this warrants further investigation.

In this work, we have not investigated whether HCV replication occurs after fusion of other PBMCs with Huh7.5 cells. HCV replication has been reported in patient-

derived B and T lymphocytes as well as monocytes/macrophages [116, 132, 133, 324]. T cells can be infected with patient-derived HCV *in vitro* [124], whilst B cells can be used to infect hepatoma cells with cell culture-produced HCV (HCVcc) [130]. Although this work has focussed on monocytes/macrophages, extrahepatic replication in other PBMCs may also be clinically important.

6.5 Concluding remarks

The work in this thesis has demonstrated that viable HCV RNA resides within patient-derived monocytes. By fusing these cells with the replication-permissive cell line Huh7.5, low level viral replication can be induced. Although the level of replication is low, it is sufficient to be detected using a sensitive PCR assay and can be reproducibly inhibited by antiviral drugs. Pre-stimulated THP-1 can be infected with HCV derived from patient-serum, and low level replication of this virus was seen after fusion of infected THP-1 with Huh7.5 cells. This could be inhibited by a range of antiviral drugs with diverse mechanisms of action in a genotype-specific and patient-specific manner.

HCV infection of monocytes has implications for several clinical aspects of chronic HCV infection, including relapse after therapy. A limiting factor of our viral replication model is low level viral replication in the fused cells and this has hampered our attempts to investigate mechanisms underlying HCV entry into monocytes and replication after fusion. Our work in this area continues as the biology of HCV replication in extrahepatic sites has implications for relapse after antiviral therapy, which remains important clinically in the emerging era of DAA therapy. Greater understanding of mechanisms underlying replication in fused

monocytes:hepatocytes may allow refinement of the capture-fusion technique to enhance its suitability as a high-throughput screening tool for antiviral drug sensitivity. In turn, this may help tailor antiviral therapy to the individual to achieve the ultimate treatment goal in HCV: a cure for all.

7.0 References

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Appendix A

Sample	Subtype	Age (Years)	Gender	VL (IU/mL)	ALT	Fibrosis score	Ethnicity	Treatment status
SER101	1a	69	M	15488166	43	5	White British	prev NR, subsequent SVR with telaprevir
SER102	1a	61	F	15849	12	1	White British	previous NR, SVR in alisporivir trial
SER103	1a	67	M	1318257	82	2	White British	naïve
SER104	1b	63	F	1778279	50	2	White - other background	naïve (subsequent relapse)
SER105	1a	54	M	1332903	96	1	White British	NRx2
SER106	1a	45	M	3000000	126	1	White British	naïve
SER107	1a	57	M	2701351	54	2	White British	naïve
SER108	1a	34	M	3636300	62	N/A	White other	relapse in PI/NS5B trial
SER109	1b	53	M	207337	66	N/A	White other	naïve (subsequent relapse)
SER110	1b	59	M	851857	51	6	White British	naïve
SER111	1a	57	M	7075272	56	2	White British	naïve (subsequent SVR in sofosbuvir/5855/RBV trial)
SER112	1a	38	M	3356395	67	3	White other	naïve
SER113	1a	56	F	1548816	280	1	White British	naïve
SER114	1a	58	M	300333	24	3	Other	prev NR; breakthrough on IFN/RBV/boceprevir
SER115	1a	61	M	2470464	131	N/A	White British	prev relapse; subsequent SVR with telaprevir
SER116	1b	64	M	417312	71	6	White British	naïve, SVR on Abbvie trial
SER117	1a	44	F	742292	38	1	Asian or Asian British - Pakistani	previous partial responder to IFN/RBV
SER118	1b	63	M	494740	90	4	White British	naïve, subsequent relapse IFN/RBV/telaprevir
SER119	1a	59	F	2042201	168	6	White British	naïve
SER120	1a	46	M	5186029	99	5	White British	NR to IFN/RBV and to IFN/RBV/danoprevir
SER121	1a	63	F	1243820	18	6	White British	NR to IFN/RBV and to IFN/RBV/telaprevir
SER122	1a	55	M	3890451	47	2	White British	relapse, subsequent SVR with telaprevir
SER123	1b	51	F	5495409	30	2	Asian or Asian British - Bangladeshi	SVR
SER124	1a	54	M	3467369	167	4	White British	SVR
SER125	1a	45	M	794328.2	56	3	White - other background	NR
SER126	1a	58	F	14125375	33	4	White British	NR

Table A-1. Clinical features of patients with genotype 1 HCV who supplied serum samples for use in capture-fusion experiments. VL, viral load; ALT, alanine transaminase; NR, non-response to previous treatment with pegylated interferon and ribavirin; SVR, sustained virological response; PI, protease inhibitor; NS5B, antiviral drug targeting HCV NS5B; IFN, pegylated interferon; RBV, ribavirin; N/A, not available.

Sample	Subtype	Age (Years)	Gender	VL (IU/mL)	ALT	Fibrosis score	Ethnicity	Treatment status
SER201	2b	53	M	1636987	154	N/A	White British	naïve
SER202	2	49	M	2884032	100	6	Black - other	NR
SER203	2b	59	M	258301	253	N/A	White British	naïve, subsequent SVR
SER204	2	54	M	2436479	22	6	White British	naïve

Table A-2. Clinical features of patients with genotype 2 HCV who supplied serum samples for use in capture-fusion experiments. VL, viral load; ALT, alanine transaminase; NR, non-response to previous treatment with pegylated interferon and ribavirin; SVR, sustained virological response; N/A, not available.

Sample	Subtype	Age (Years)	Gender	VL (IU/mL)	ALT	Fibrosis score	Ethnicity	Treatment status
SER301	3a	47	M	12589254	140	1	White British	naïve (subsequent SVR)
SER302	3a	58	M	5888437	72	4	White British	relapse
SER303	3a	43	M	2290868	153	5	Asian or Asian British - Pakistani	naïve (subsequent relapse)
SER304	3a	40	M	3890451	152	5	Asian or Asian British - Pakistani	relapse
SER305	3a	58	F	12384588	99	N/A	Asian or Asian British - Pakistani	naïve (subsequent NR to IFN/RBV)
SER306	3a	24	M	170663	101	3	Asian or Asian British - Pakistani	naïve (subsequent SVR)
SER307	3a	26	M	282092	165	2	Other	naïve (subsequent SVR)
SER308	3a	53	M	2879840	88	2	White British	naïve
SER309	3a	41	F	3598151	51	1	Asian - Other	naïve (subsequent SVR)
SER310	3a	58	M	177526	168	5	White British	naïve (subsequent relapse)
SER311	3a	58	M	4168694	117	2	White British	naïve
SER312	3a	23	M	15586	114	N/A	Asian or Asian British - Pakistani	naïve, subsequent SVR
SER313	3a	40	F	282608	109	N/A	Asian - Other	naïve (subsequent SVR)
SER314	3b	42	M	197106	161	2	Other	naïve
SER315	3a	61	F	1699009	76	6	White British	discontinued Rx - decompensation
SER316	3a	54	M	5970686	171		Asian - other	relapse
SER317	3a	53	F	149882	29	1	White British	relapse
SER318	3b	75	M	3715352	66	6	Asian or Asian British - Bangladeshi	relapse
SER319	3a	38	F	177828	58	5	Asian - other	SVR
SER320	3b	59	F	281838	91	5	Asian or Asian British - Pakistani	SVR
SER321	3b	53	M	1621810	43	4	Asian or Asian British - Bangladeshi	SVR
SER322	3a	58	M	1153109	179	6	White British	Relapse
SER323	3a	48	M	4134234	216	6	Asian or Asian British - Pakistani	Relapse
SER324	3a	49	F	1945911	52	6	Asian or Asian British - Pakistani	Relapse
SER325	3a	52	M	366120	254	5	Asian or Asian British - Pakistani	SVR
SER326	3a	46	M	546020	151	4	White British	SVR
SER327	3a	56	F	1776799	47	6	Other	SVR

Table A-3. Clinical features of patients with genotype 3 HCV who supplied serum samples for use in capture-fusion experiments. VL, viral load; ALT, alanine transaminase; NR, non-response to previous treatment with pegylated interferon and ribavirin; SVR, sustained virological response; IFN, pegylated interferon; RBV, ribavirin; N/A, not available.

Sample	Subtype	Age (Years)	Gender	VL (IU/mL)	ALT	Fibrosis score	Ethnicity	Treatment status
SER401	4	44	F	618730	88	N/A	Black or Black British - African	Previous NR
SER402	4	47	F	4555	50	0	Black - other	Previous NR
SER403	4	48	F	283015	36	0	White - other	naive

Table A-4. Clinical features of patients with genotype 4 HCV who supplied serum samples for use in capture-fusion experiments. VL, viral load; ALT, alanine transaminase; NR, non-response to previous treatment with pegylated interferon and ribavirin; N/A, not available.

Sample	Subtype	Age (Years)	Gender	VL	ALT	Fibrosis score	Ethnicity	Treatment status
SER601	6	33	F	564798	N/A	N/A	Asian - other	naive
SER602	6	41	M	2398833	14	N/A	Asian - other	Naïve (subsequent SVR)
SER603	6k	62	M	939746	147	1	Other - Chinese	naïve

Table A-5. Clinical features of patients with genotype 6 HCV who supplied serum samples for use in capture-fusion experiments. VL, viral load; ALT, alanine transaminase; SVR, sustained virological response to treatment; N/A, not available.

Appendix B. Communications arising from this research

Development and Validation of a “Capture-Fusion” Model to Study Drug Sensitivity of Patient-Derived Hepatitis C

Morven E. Cunningham, Alia Javaid, Jenny Waters, Joseph Davidson-Wright, Joshua L.C. Wong, Meleri Jones, and Graham R. Foster

Emerging therapies for chronic hepatitis C viral (HCV) infection involve inhibition of viral enzymes with drug combinations. Natural, or treatment-induced, enzyme polymorphisms reduce efficacy. We developed a phenotyping assay to aid drug selection based on viral transfer from monocytes to hepatocytes. We studied HCV in monocytes from infected patients and developed a model in which patient-derived HCV is “captured” by the cell line THP-1 and replication assessed after fusion to hepatoma cells. We found that monocytes from HCV-infected patients harbor virus that replicates when cells are fused to hepatocytes. THP-1 cells incubated with infected sera capture HCV, which replicates when fused to hepatocytes. Inhibitable replication of all HCV genotypes was achieved (42 of 52 isolates). We measured sensitivity of telaprevir (TVR) and alisporivir (AVR) in different genotypes, and showed differences in 50% inhibitory concentration (IC₅₀) correlating with clinical response (TVR IC₅₀ for genotype (G)1 was 0.042 ± 0.003 vs. 0.117 ± 0.015 μ M for G3, whereas AVR IC₅₀ for G1 was 0.139 ± 0.013 vs. 0.044 ± 0.007 μ M for G3). We tested TVR-resistant viral isolates and identified changes in IC₅₀. One patient with a poor clinical response to TVR and wild-type viral sequence showed reduced TVR sensitivity in our assay. We studied samples from a 2-week TVR monotherapy study in which 5 of 8 patients with G3 HCV did not respond whereas 3 of 8 patients did. The “capture-fusion” assay correctly identified responders. **Conclusion:** The capture-fusion model represents a promising new technique that may help identify appropriate treatment strategies for patients with chronic HCV infection. (HEPATOLOGY 2015;61:1192-1204)

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Therapy for patients infected with hepatitis C virus (HCV) is rapidly evolving, and interferon (IFN) and ribavirin (RBV) are being replaced by direct-acting antiviral (DAA) agents.¹ Drugs against the viral nonstructural protein 3 of HCV (NS3) protease (telaprevir [TVR] simeprevir,

and boceprevir) and nonstructural protein 5B of HCV (NS5B) polymerase (sofosbuvir; SOF) have been licensed, and new protease, nonstructural protein 5A of HCV (NS5A), and polymerase inhibitors are in clinical trials. In combination with IFN, most drugs increase the proportion of patients who respond. This has led to attempts to avoid IFN-related side effects by combining drugs of different classes in IFN-free regimens. Distinct viral strains respond differently, for

Abbreviations: Abs, antibodies; AVR, alisporivir; DAA, direct acting antiviral; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; G, genotype; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JFH-1, Japanese fulminant hepatitis type 1; HCV, hepatitis C virus; IC₅₀, 50% inhibitory concentration; IF, immunofluorescence; IFN, interferon; IL, interleukin; mRNA, messenger RNA; NS3, nonstructural protein 3 of the hepatitis C virus; NS5A, nonstructural protein 5A of the hepatitis C virus; NS5B, nonstructural protein 5B of the hepatitis C virus; PBMCs, peripheral blood mononuclear cells; PEG, polyethylene glycol; Peg-IFN, pegylated IFN; PI3K/Pi4K, phosphatidylinositol 3- and 4-kinase; PMA, phorbol 12-myristate 13-acetate; PS, penicillin/streptomycin; RBV, ribavirin; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SOF, sofosbuvir; SR-B1, scavenger receptor class B type 1; SVR, sustained virological response; TVR, telaprevir; WT, wild type.

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example, genotype (G)1b strains respond well to protease inhibitors combined with non-nucleotide polymerase inhibitors or NS5A inhibitors,^{2,3} but G1a strains require additional drugs.⁴ Some G1a strains do respond to protease plus non-nucleoside drugs,⁵ but their characteristics are undefined. IFN-free SOF is effective for G2 HCV and has some efficacy in G1, but less so in G3.⁶

Development of DAAs was facilitated by *in vitro* HCV replication systems (the G1 replicon and replication-permissive G2 strain, Japanese fulminant hepatitis type 1 [JFH-1])^{7,8} that allowed high-throughput screening. Subgenomic replicons based around G3a and G4a have extended the range of genotypes that can be studied,^{9,10} and chimeric variants have been used to analyze resistance-associated variants. These systems are of limited value in analysis of a patient's viral sensitivity—defining drug sensitivity of a viral isolate requires subcloning each DAA target gene and insertion into a replicon. Difficulty in generating replicons with more than one modified locus precludes studies of multidrug combinations. In human immunodeficiency virus infection, viral phenotyping assays enabled selection of optimal regimens, although progress in genotypic resistance testing has reduced their value. For G1 HCV, some viral motifs associated with protease inhibitor treatment failure have been identified,¹¹ but for other genotypes there is no association between sequence and response,¹² indicating that, in the short term, viral phenotyping assays may be useful in identifying optimal regimens for less-responsive viral strains. Although recently presented data indicate that emerging drug regimens cure over 90% of patients with G1 HCV,^{13,14} the proportion of patients who fail may be higher in practice. To avoid development of “multidrug-resistant” HCV strains, careful selection of “second-line” regimens will be required. Identification of the most effective treatment regimens in this scenario would be facilitated by phenotyping assays.

Association of HCV with extrahepatic sites, such as peripheral blood mononuclear cells (PBMCs), is established, although extrahepatic viral replication remains controversial.^{15–20} Here, we describe a “capture-fusion” technique in which patient-derived HCV is “captured”

by monocytes and transferred to hepatocytes using cell fusion. This allows analysis of patient-derived virus from all genotypes and drug sensitivity and treatment response can be predicted. This new assay may be helpful in determining optimal drug combinations and be of particular value in patients who have failed “first-line” regimens.

Materials and Methods

Cells, Reagents, and Clinical Material. Huh7.5 cells (kindly provided by C.M. Rice, Rockefeller Institute, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM) with glutamine, 10% fetal calf serum (FCS), and 1% penicillin/streptomycin (DMEM/10% FCS/PS). THP-1 cells²¹ were maintained in RPMI with glutamine, 10% FCS, and 1% PS.

PBMCs and sera were obtained from consenting patients with chronic HCV infection.

Stimulants/inhibitors used were phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Dorset, UK), IFN- γ (Invitrogen, Paisley, UK), and wortmannin (Sigma-Aldrich).

TVR and alisporivir (AVR) were kindly supplied by Janssen Virology (Baar, Switzerland) and Novartis Pharma AG (Basel, Switzerland), respectively. IFN- α -2a was from Cambridge Bioscience (Cambridge, UK) and RBV from Sigma-Aldrich. Ethical approval for the study was given by London-City Research Ethics Committee, and informed consent obtained from all patients. The study was conducted in accord with the Declaration of Helsinki.

Fusion of Patient Monocytes. PBMCs were separated from whole blood by centrifugation on Ficoll-Paque (GE Healthcare, Buckinghamshire, UK). The PBMC layer was washed twice before positive selection of CD14⁺ cells by magnetic separation (Milteyi Biotec, Surrey, UK), according to manufacturer's instructions. For fusion optimization experiments, CD14⁺ cells were incubated with DiI and Huh7.5 cells with DiO (Life Technologies, Paisley, UK) before incubation with polyethylene glycol (PEG) 1500 (Roche Diagnostics, Burgess Hill, UK). Cells were fixed with 4% paraformaldehyde (PFA) and enumerated by flow

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cytometry (FACSCanto II; BD Biosciences, Oxford, UK).

For all subsequent fusion experiments, Huh7.5 cells and CD14⁺ cells (1:1) were incubated with PEG at 37°C for 2 minutes, then prewarmed medium (DMEM) added dropwise and the cells washed by centrifugation. Fused cells were seeded into six-well plates (5×10^5 cells/mL) and maintained at 37°C.

Stimulation and Infection of THP-1 Cells. THP-1 cells were seeded into six-well plates (10^6 cells/mL) and maintained for 18 hours, with or without IFN- γ (10 ng/mL) and PMA (200 ng/mL). Cells were washed thrice and medium replaced with RPMI/2% FCS and patient serum (1 HCV IU/cell). After incubation (37°C for 18–24 hours), supernatant was removed and cells washed. Adherent cells were removed by scraping and combined with Huh7.5 cells (1:1). Cell fusion was performed as described above. Fused cells were seeded into six-well plates (10^5 cells/mL) and maintained at 37°C with or without drugs for up to 7 days. In selected experiments, supernatants were pooled from non-drug-treated wells and concentrated by filtration through a 0.45- μ m filter, then 10 mL was layered on 4 mL of 20% sucrose. Supernatants were centrifuged (24,000g) for 2 hours and the pellet resuspended in 1 mL of RPMI. For capture-fusion experiments using concentrated supernatant, 1×10^6 prestimulated THP-1 cells were incubated with 1 mL of concentrated supernatant for 24 hours before fusion.

Drug Inhibition Assays. Fused cells were rested overnight before the addition of drugs. TVR and AVR were diluted from 20-mM stock solutions in dimethyl sulfoxide (DMSO). Each drug concentration was tested in quadruplicate. Drug dilution mix alone (RPMI/2% FCS/0.5% DMSO) was added to control wells. Media and drug were refreshed at day 3. Viral RNA was calculated as a percentage of that in untreated wells. Dose-response curves were constructed and used to estimate the 50% inhibitory concentration (IC₅₀) of drug using Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA).

Cell Viability Assays. Cell viability was measured using the Cell Titer-Glo assay (Promega, Southampton, UK) and a FLUOstar Optima plate reader (BMG Labtech, Aylesbury, UK), according to the manufacturer's instructions.

Quantification of HCV RNA and Gene Expression. Total RNA was extracted with TRIzol (Invitrogen) and quantified using RiboGreen (Invitrogen), according to the manufacturer's instructions. Complementary DNA was synthesised using Moloney murine

leukemia virus reverse transcriptase (Promega) and diluted (1:10) for amplification by quantitative polymerase chain reaction (qPCR; QuantiTect SYBR Green PCR kit; Qiagen, Hilden, Germany). For primers and cycling conditions, see the Supporting Information. Target messenger RNA (mRNA) was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin expression, which were confirmed to be stably expressed in these experiments (GeNorm; Primer-Design, Southampton, UK).

For HCV copy number quantification, one-step reverse-transcription PCR (RT-qPCR) using the QuantiTect Virus Kit (Qiagen) and TaqMan Gene Expression Assay HCV primer and probe (Applied Biosystems, Paisley, UK) was performed. Serial dilutions of an RNA standard were included in each PCR run and results expressed relative to total sample RNA.

Fluorescence-Activated Cell-Sorting Analysis. Cells were incubated with primary antibody (Ab) or appropriate isotype control in 1% bovine serum albumin/10% FCS for 30 minutes. Alexa Fluor 488 secondary Abs were used to detect bound primary Ab and quantified on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreesStar, Ashland, OR).

Statistical Analysis. Data were tested for normality using the D'Agostino's and Pearson's test. Statistical analyses used the Student *t* test for parametric data or Mann-Whitney's U test for nonparametric data, with *P* < 0.05 considered significant.

Results

Identification of Replication-Competent HCV in Patient-Derived Monocytes by Fusion With Huh7.5 Cells. We examined CD14(+) monocytes from patients with chronic HCV infection (N = 4) and found low levels of HCV that did not increase during cell culture (7 days), implying a lack of viral replication (data not shown). We then fused patient-derived monocytes with Huh7.5 cells using PEG. Dye-labeled cells were used to determine optimal fusion conditions. Fusion of 40% of CD14(+) monocytes with Huh7.5 cells was achieved using optimized conditions (see Materials and Methods; Fig. 1A). Monocytes from patients infected with diverse viral genotypes were fused to Huh7.5 cells and maintained in culture for up to 21 days. HCV RNA increased progressively up to 7 days after fusion, but declined thereafter (Fig. 1B). In these preliminary experiments, HCV RNA was quantified relative to beta-actin expression. To confirm reliable detection of low-level HCV RNA and quantify

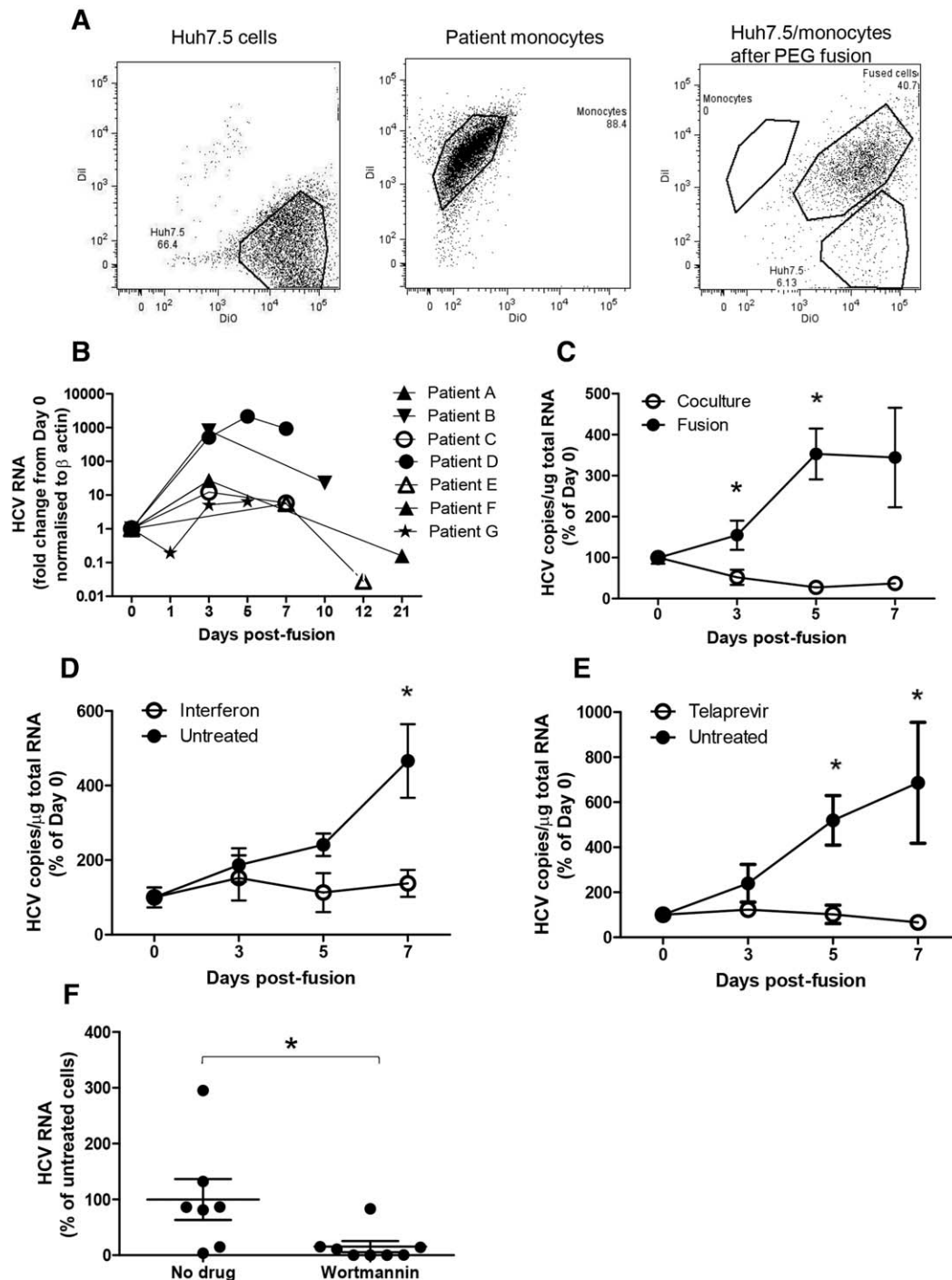


Fig. 1. Detectable HCV RNA increases after fusion of patient-derived monocytes with Huh7.5 cells, and this can be inhibited with antiviral drugs. (A) Fluorescence-activated cell-sorting plot showing PEG-mediated fusion of patient monocytes and Huh7.5 cells. Huh7.5 cells were stained with DiO and CD14(+) monocytes with DiI. After PEG fusion, the percentage of cells expressing both dyes was enumerated by flow cytometry. Figures refer to percentage of total cells. (B) monocytes from 7 patients with chronic HCV were fused with Huh7.5 and HCV RNA quantified at various times up to 21 days after fusion. Not all time points are available for all samples as a result of limited cell numbers. Results are expressed as fold change from unfused monocytes and normalized to beta-actin expression. (C) Mean \pm standard error of the mean (SEM) of five further experiments where monocytes from HCV patients were either fused or cocultured with Huh7.5 cells and HCV RNA quantified up to 7 days after fusion. $P = 0.038$ at 3 days and $P = 0.015$ at 5 days postfusion for the comparison between fused and unfused cells. (D) Mean \pm SEM of two further experiments where fused patient monocytes were cultured with or without 100 IU/mL of IFN- α -2a. $P = 0.031$ for the comparison between treated and untreated cells at day 7 postfusion. (E) Mean \pm SEM of three further experiments where fused G1 patient monocytes were cultured with or without TVR 0.5 μ M. $P = 0.0001$ at 5 days and $P = 0.0002$ at 7 days postfusion for the comparison between treated and untreated cells. (F) Mean \pm SEM of two further experiments where fused patient monocytes were cultured with or without the PI3K/PI4K inhibitor, wortmannin, 0.5 μ M for 5 days after fusion. $P = 0.014$ for the comparison between treated and untreated cells. (C-E) HCV copies/ μ g total RNA as a percentage of day 0 postfusion to normalize for differences in HCV-RNA yield between individual patient samples. (F) HCV copies/ μ g total RNA as a percentage of untreated cells at day 5 postfusion.

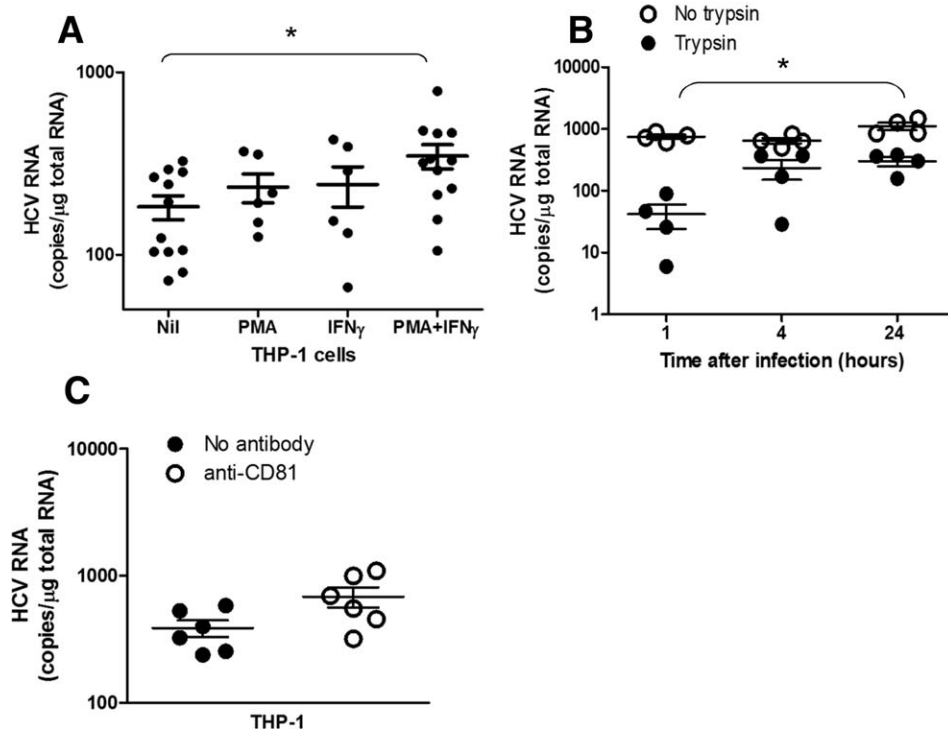


Fig. 2. Association of patient-derived HCV with THP-1 is enhanced by PMA/IFN- γ , but is independent of CD81. (A) THP-1 cells were cultured for 24 hours with PMA 200 ng/mL, IFN- γ 10 ng/mL, PMA 200 ng/mL and IFN- γ 10 ng/mL or no stimulants before incubation with patient serum. HCV RNA was measured 24 hours after infection. $P = 0.026$ for the comparison between unstimulated and PMA/IFN- γ -stimulated cells. (B) THP-1 cells prestimulated with PMA/IFN- γ were treated with trypsin at various times after incubation with patient sera. $P = 0.029$ for the comparison between trypsin treatment 0 and 24 hours postinfection. (C) PMA/IFN- γ -stimulated THP-1 cells were incubated in the presence or absence of anti-CD81 blocking Ab (5 μ g/mL) for 1 hour before infection with patient-derived HCV. HCV RNA was quantified after 24 hours. Graph shows mean \pm standard error of the mean of two independent experiments. $P = 0.09$ for the comparison between antibody treated and untreated cells.

HCV-RNA copy number, a one-step RT-qPCR assay was employed (see Materials and Methods) with a quantification range of 10 - 10^8 HCV copies per reaction (Supporting Fig. 1). Rather than logarithmic increases in HCV RNA observed in propagation of cell-culture HCV strains, we typically observed a 2- to 10-fold increase in viral RNA over 7 days after fusion, suggesting low-level replication (Fig. 1C). Treatment of fused cells with antiviral drugs abolished this increase (Fig. 1D,E). Treatment of fused cells with wortmannin, a phosphatidylinositol 3- and 4-kinase (PI3K/PI4K) inhibitor, also reduced replication after fusion (Fig. 1F). Contamination by laboratory HCV strains was excluded by viral sequencing (data not shown). Attempts to demonstrate HCV replication using less-sensitive techniques (western blotting and indirect immunofluorescence [IF]) were unsuccessful, most likely because of the low level of viral replication (data not shown). Together, these results indicate that viable HCV is associated with monocytes of patients with chronic HCV infection. Fusion of infected monocytes with Huh7.5 cells permits sufficient viral replication to be detected by a sensitive PCR assay.

Association of Patient-Derived HCV With THP-1 Cells Is Enhanced by PMA and IFN- γ , but Is Independent of CD81. Patient-derived HCV in fused cells can be inhibited with antiviral drugs, allowing identification of patient-specific responses to antiviral therapies. Given the limitations of working with patient monocytes, we examined whether cultured monocytes could be used to capture HCV from patient serum. After incubation of the monocytic cell line, THP-1, with patient serum, we found little association of HCV with THP-1 cells. Others report that PMA and IFN- γ pretreatment enhances HCV uptake into monocytic cell lines.²² Stimulation of THP-1 with PMA/IFN- γ enhanced the association of patient-derived HCV (Fig. 2A), and trypsin treatment of virus-loaded THP-1 cells after 4 hours reduced viral levels, indicating that virus was internalized between 1 and 4 hours after infection (Fig. 2B).

HCV entry into hepatocytes is dependent upon CD81, scavenger receptor class B type 1 (SR-B1), claudin, and occludin.²³ Neither mRNA nor cell-surface expression of CD81 or SR-B1 was enhanced by PMA/IFN- γ treatment of THP-1 (Supporting Fig.

2). Claudin-1 and occludin were not expressed by these cells. Blocking Abs to CD81 did not reduce HCV association with stimulated THP-1 (Fig. 2C), although JFH-1 entry into Huh7.5 cells was inhibited (Supporting Fig. 3). These data suggest that HCV association with THP-1 is independent of CD81 and other receptors are associated with HCV entry into hepatocytes.

Accumulation of Patient-Derived HCV Occurs After Capture Fusion. Accumulation of HCV RNA was observed after Huh7.5 fusion to PMA/IFN- γ -stimulated, HCV-exposed THP-1 cells, compared to HCV-exposed, PEG-treated Huh7.5, THP-1, or Huh7.5 alone (Fig. 3A). HCV-RNA accumulation after fusion was significantly greater when THP-1 were prestimulated, particularly when IFN- γ was added to PMA stimulation (Fig. 3B). To examine the potential antiviral effects of IFN- γ , we studied expression of the IFN-inducible gene MxA. MxA expression was not induced by cell fusion and was low in fusions with HCV-infected monocytes, even when the monocytes had been pretreated with PMA/IFN- γ , but could be up-regulated by subsequent treatment with IFN- α (Fig. 3C). Priming of Huh7.5 cells with IFN- α significantly reduced HCV replication after fusion (Fig. 3D). Thus, addition of IFN- γ increases uptake of virus by monocytes, but fusing IFN- γ -exposed cells does not lead to induction of antiviral genes in fused cells. However, direct stimulation of Huh7.5 cells with the more potent antiviral IFN, IFN- α , does reduce HCV replication. These data suggest PMA/IFN- γ treatment of THP-1 is important for HCV capture, but does not trigger induction of innate antiviral responses, although the cells are still able to induce antiviral genes.

To establish whether full replication cycles occurred in the fused cells with release of infectious virus, supernatants from capture-fusion experiments were collected, concentrated, and applied to Huh7.5 cells or to stimulated THP-1 cells in a fresh capture-fusion cycle. HCV RNA accumulated over time in fused cells and to a much lesser extent in Huh7.5 cells (Fig. 3E). These data suggest that HCV undergoes replicative cycles with release of infectious virions in the capture-fusion model.

HCV RNA Accumulation Occurs After Fusion in Isolates From All Viral Genotypes and Can Be Inhibited by Antiviral Drugs. Fifty-two viral isolates of genotypes 1-6 were tested in this capture-fusion assay and HCV RNA could be quantified (>10 copies per reaction) after fusion in 42 (81%). Median viral yield was 63 copies/ μ g total RNA (range, 14-354)

equivalent to median 19 HCV copies per fused cell (range, 4-106). Of the remaining 10 samples, seven were archival (3-7 years old) and may have degraded during storage. Three samples produced detectable HCV RNA below the quantification limit in repeated experiments. HCV-RNA yield varied between samples, but did not relate to viral load or disease activity (Supporting Fig. 4), and was consistent in repeated experiments for a given sample (Supporting Fig. 5), suggesting that replicative fitness in this model is influenced by intrinsic viral characteristics. TVR exposure after fusion caused a dose-dependent reduction in HCV RNA in isolates from all genotypes except G3, known to be poorly TVR sensitive (Fig. 3F). Experiments with alternative inhibitors confirmed that sensitivity of G3 HCV could also be studied (see below).

Drug Sensitivity of Patient-Derived HCV in the Capture-Fusion Model Reflects Genotypic Patterns of Response to Antiviral Drugs. We observed that G3 HCV was poorly inhibited by TVR in the capture-fusion assay (Fig. 3F). TVR inhibits G1, but has little effect against G3 HCV.²⁴⁻²⁶ Conversely, the cyclophilin inhibitor, AVR, is more efficacious against G2 and G3 than G1 and G4.²⁷ THP-1 cells exposed to sera from G1- and G3-infected donors were fused with Huh7.5, then treated with TVR or AVR for 5 days before quantification of HCV RNA. Dose-response curves were constructed and used to calculate an IC₅₀. Figure 4 shows pooled results from 14 donors, normalized to untreated cells. Dose-response curves were constructed for all isolates which reached quantifiable levels (Supporting Fig. 6 shows absolute values for representative patients). Drug-induced cytotoxicity was excluded by measuring cell viability after drug exposure. TVR had no effect on cell survival. The minor effects of doses of AVR >0.1 μ M were insufficient to account for the reduction in HCV RNA (Supporting Fig. 7). G1 samples were significantly more sensitive to TVR and less sensitive to AVR than G3 (Fig. 4A-D). Individual TVR and AVR IC₅₀s for each sample are shown in Fig. 4E,F. G2 isolates were sensitive to TVR and AVR, whereas G4 strains showed a similar AVR response to G1 (in keeping with clinical trial data). Some G4 isolates were poorly sensitive to TVR, consistent with clinical trial findings.²⁸ However, other G4 isolates were inhibited by TVR in the assay, suggesting differing TVR susceptibility amongst G4 strains (Supporting Fig. 8).

Drug Sensitivity in the Capture-Fusion Assay Reflects Phenotypic Treatment Response. To explore whether clinical TVR resistance could be detected in the capture-fusion assay, pretreatment and post-

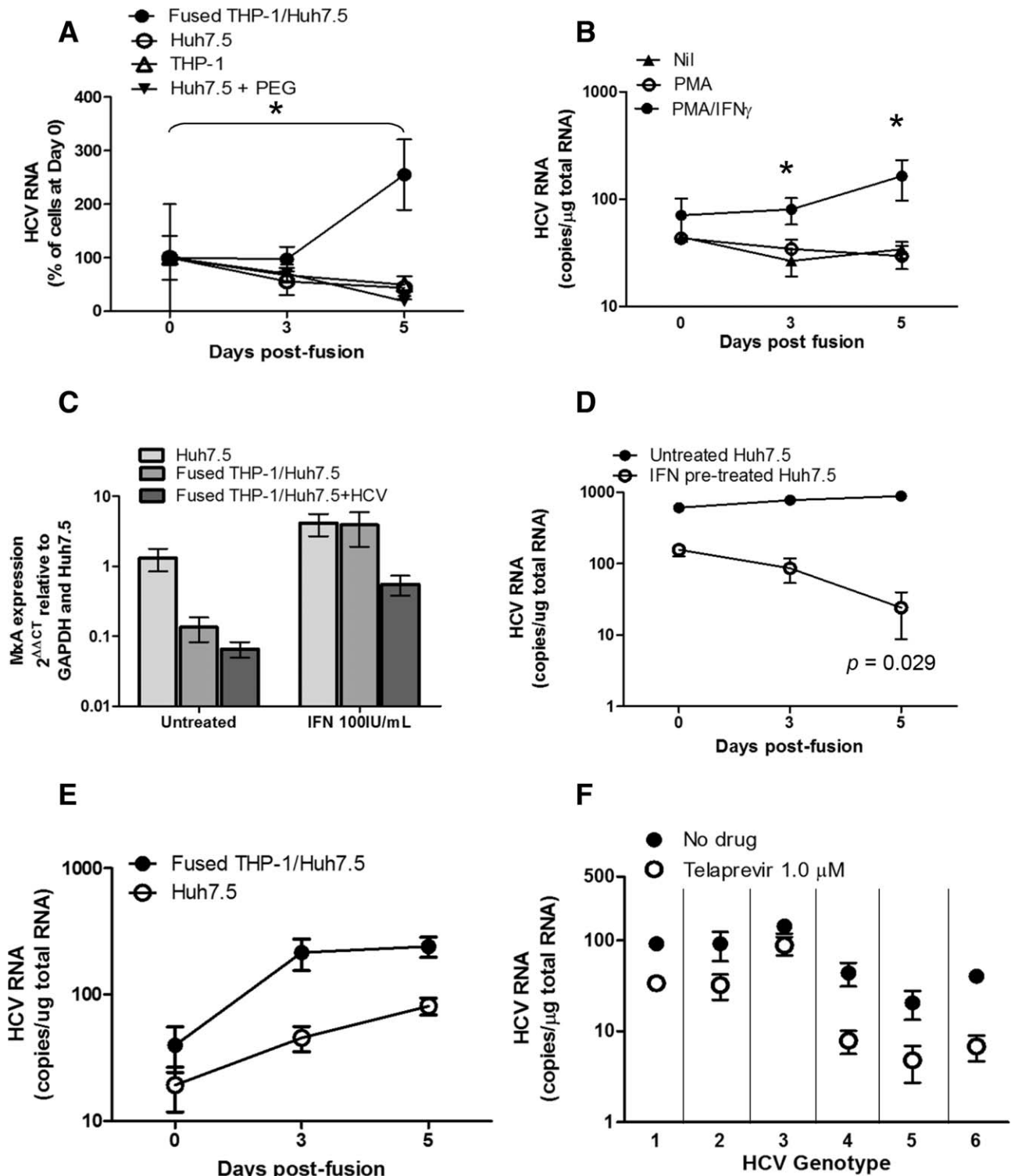


Fig. 3. Replication of patient-derived HCV occurs after capture by prestimulated THP-1 and fusion with Huh7.5 cells and can be inhibited by telaprevir. A. Mean \pm standard error of the mean (SEM) of two independent experiments where HCV RNA was quantified from PMA/IFN- γ -stimulated, HCV-exposed THP-1 fused with Huh7.5 cells, from HCV-exposed THP-1 and Huh7.5 cells without fusion, and from HCV-exposed Huh7.5 cells treated with PEG (fusion protocol). $P = 0.019$ for the comparison between HCV RNA in fused THP-1/Huh7.5 at days and 5 postfusion. Results are shown as percentage of HCV RNA in cells at day 0. (B) Representative of two independent experiments where THP-1 cells were cultured with PMA 200 ng/mL, PMA 200 ng/mL and IFN- γ 10 ng/mL or no stimulants for 24 hours before infection with patient-derived HCV and fusion with Huh7.5 cells. HCV RNA was quantified up to 5 days after fusion. $P = 0.005$ and $P = 0.015$ for the comparison between unstimulated and PMA/IFN- γ -stimulated THP-1 at days 3 and 5 postfusion, respectively. (C) PMA/IFN- γ -treated THP-1 fused with Huh7.5 (labeled fused THP-1/Huh7.5), PMA/IFN- γ -treated, HCV-exposed THP-1 fused with Huh7.5 (labeled fused THP-1/Huh7.5 + HCV), or unfused Huh7.5 (labeled Huh7.5) were cultured for 5 days in the presence or absence of IFN- α -2a (100 IU/mL). MxA mRNA was quantified by PCR and expressed relative to GAPDH expression and uninfected, unfused, untreated Huh7.5. Results are shown on a logarithmic scale. (D) Huh7.5 cells were pre-treated with IFN- α -2a (100 IU/mL) before fusion with prestimulated, HCV-infected THP-1. HCV RNA was quantified up to 5 days after fusion. (E) Mean \pm SEM of two independent experiments in which supernatant from capture-fusion experiments was applied to PMA/IFN- γ -stimulated THP-1, which were then fused with Huh7.5 in a further round of capture-fusion, or naïve Huh7.5 cells. HCV RNA was quantified up to 5 days after fusion. (F) HCV RNA 5 days after fusion in capture-fusion experiments, with or without TVR 1.0 μ M using sera from donors infected with diverse HCV genotypes. Each point represents mean HCV RNA \pm SEM from at least four biological replicates from a representative patient sample.

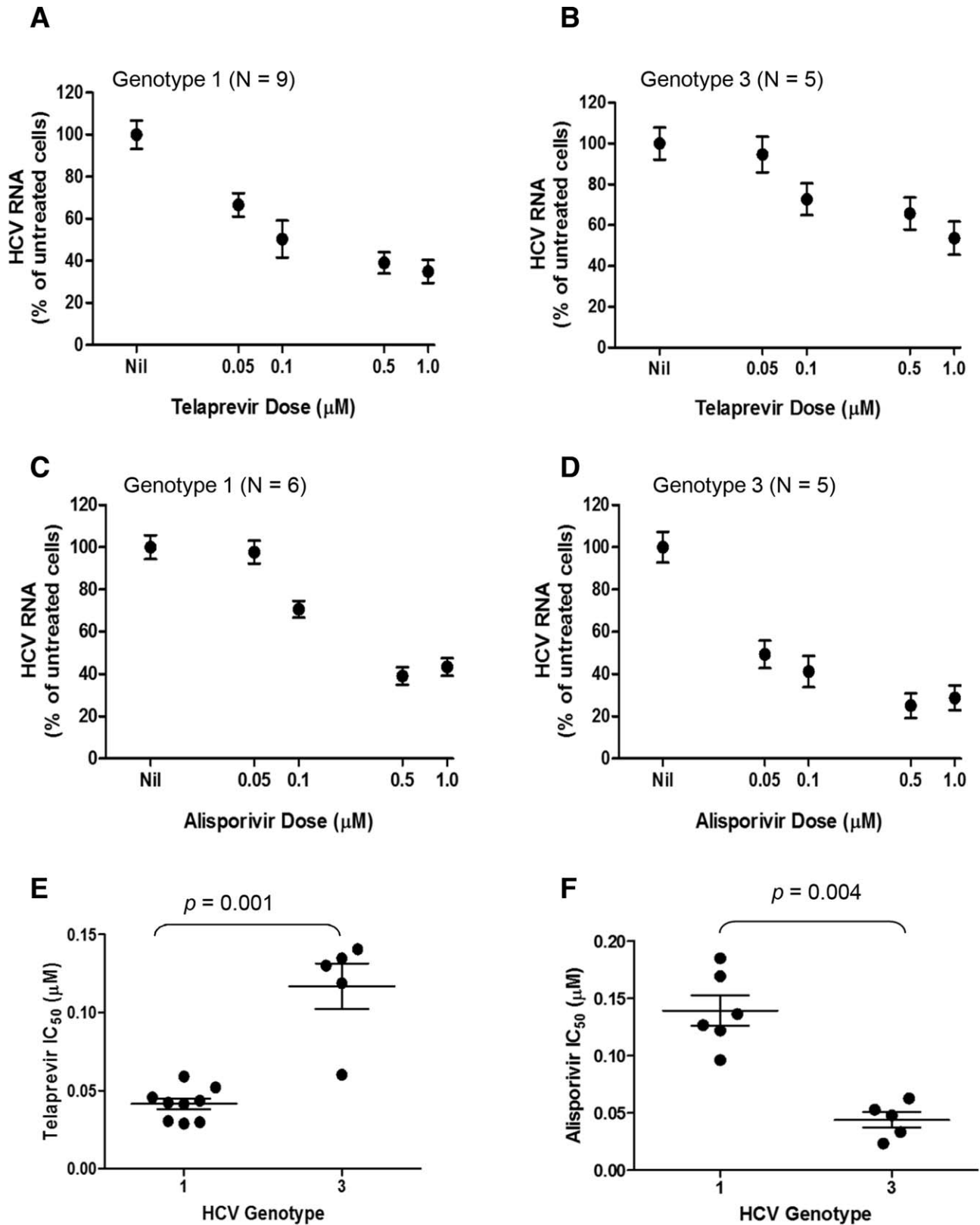


Fig. 4. Sensitivity of patient-derived HCV to antiviral drugs can be assayed in the capture-fusion model. HCV derived from serum from 9 G1 and 5 G3 donors was treated with varying concentrations of TVR or AVR in capture-fusion assays and HCV RNA measured after 5 days. G1 patient-derived HCV was more sensitive to TVR than G3 (A and B), but less sensitive to AVR (C and D). Comparison of individual IC₅₀ values showed TVR IC₅₀ was 0.042 ± 0.003 for G1 samples versus 0.117 ± 0.015 μM for G3; $P = 0.001$ (E). Conversely, AVR IC₅₀ was 0.139 ± 0.013 for G1 samples versus 0.044 ± 0.007 μM for G3; $P = 0.004$ (F). Graphs show mean \pm standard error of the mean.

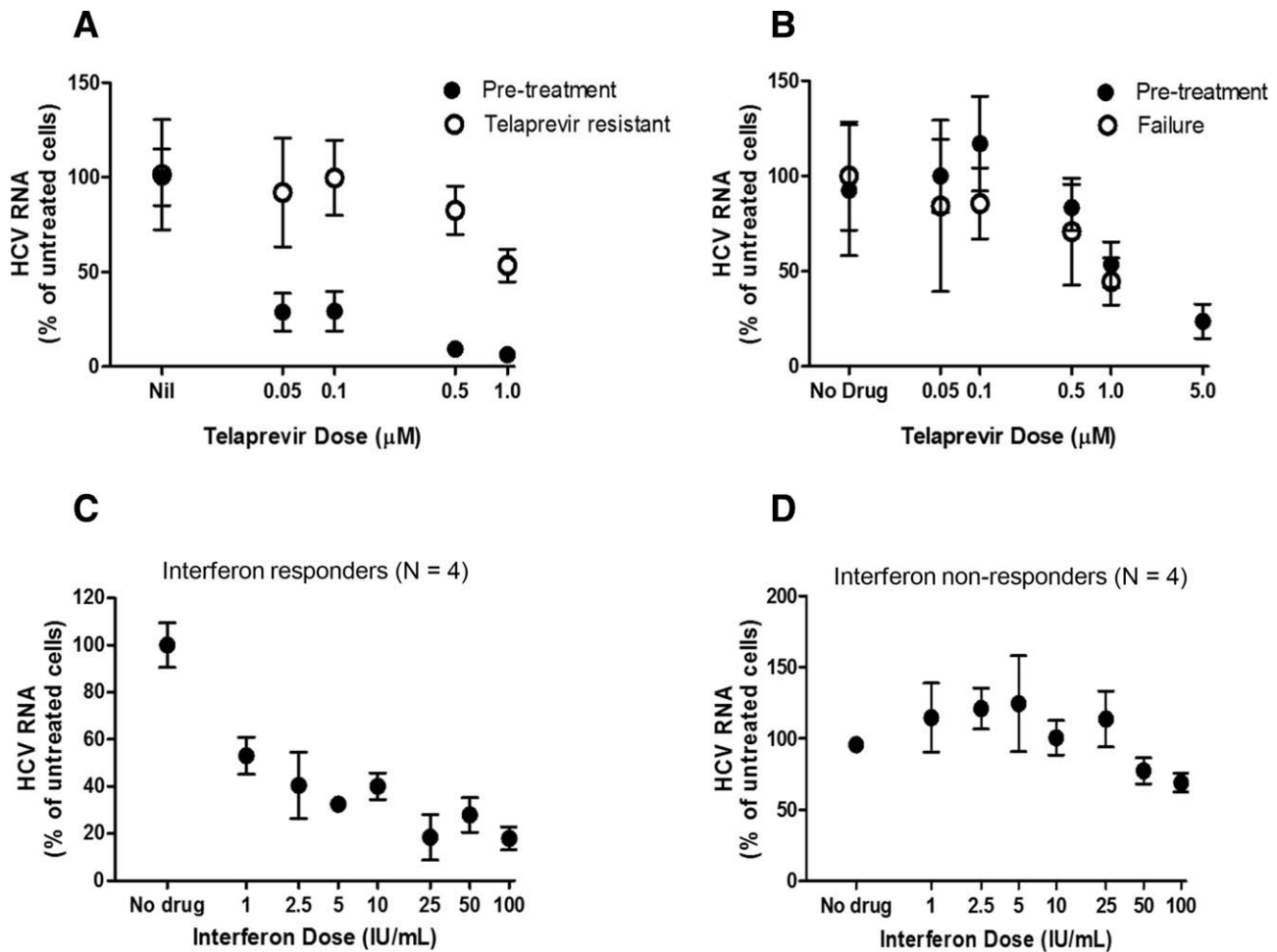


Fig. 5. Phenotypic and genotypic drug resistance correlates with drug sensitivity in the capture-fusion assay. Pretreatment and postbreakthrough sera from 2 patients who failed treatment with TVR, Peg-IFN, and RBV demonstrated loss of TVR sensitivity in the capture-fusion assay after acquisition of genotypic and clinical TVR resistance (A). Pre- and post-treatment serum from a patient with little clinical response to TVR, Peg-IFN, and RBV showed poor TVR sensitivity both pretreatment and after treatment failure (B). Using archived pretreatment sera from patients who did or did not respond to Peg-IFN and RBV treatment, HCV strains from patients who responded to treatment (C) were more sensitive to IFN in the capture-fusion assay than viral strains from patients who did not respond (D). Graphs show mean \pm standard error of the mean.

breakthrough sera were obtained from 2 patients with G1 HCV who initially responded to TVR, pegylated IFN (Peg-IFN) and RBV, but experienced virological breakthrough by treatment week 12. Sequencing of the first ~200 amino acids of viral NS3 to 1% frequency by pyrosequencing found wild-type (WT) virus at baseline and TVR resistance-associated mutations V36M/R155K at breakthrough in the majority of sequences. TVR sensitivity pre- and postbreakthrough assessed in the capture-fusion assay demonstrated a loss of sensitivity in the resistant strains (Fig. 5A). Pre- and post-treatment serum was obtained from 1 further patient with G1 HCV with a poor clinical response to TVR, Peg-IFN, and RBV (circa 1 log₁₀ reduction in viral load at week 4). No TVR resistance-associated mutations were identified pretreatment (sequencing to 1% frequency), although V36M/R155K mutations

were present at treatment failure. This strain demonstrated poor TVR sensitivity in the capture-fusion assay both before and after exposure (Fig. 5B). Pretreatment sera from 2 further patients with G1 HCV and a poor clinical response to TVR, Peg-IFN, and RBV also demonstrated poor TVR sensitivity in the capture-fusion assay (data not shown). This suggests that phenotypic sensitivity to TVR may be more predictive of response than genotypic sensitivity.

IFN sensitivity may be a determinant of acquisition of resistance to direct-acting antiviral drugs. Whether host or viral factors dominate IFN sensitivity is unclear. Archived pretreatment sera from 4 patients who responded to Peg-IFN and RBV (HCV RNA undetectable at treatment week 4) and 4 patients with a null response (<2 log₁₀ drop in viral load at treatment week 12) were used to examine IFN sensitivity

in the capture-fusion assay. Pretreatment HCV from patients who responded to IFN was more sensitive to IFN in the capture-fusion assay than virus from patients with a null response (Fig. 5C,D). Patient viral loads and viral replication achieved in the assay did not differ significantly between the groups (data not shown).

In contrast to G1, the predominant mode of treatment failure in G3 HCV infection is relapse. Relapse rates are higher in patients who receive suboptimal RBV dosing.^{29,30} Archived pretreatment sera from 10 G3 patients with cirrhosis treated with Peg-IFN and RBV, 4 with sustained virological response (SVR), and 6 who relapsed were used to study pretreatment IFN and RBV sensitivity. No difference in IFN sensitivity was observed, but pretreatment HCV from patients with SVR was more sensitive to RBV in the assay than virus from relapsers (Fig. 6).

Sensitivity to TVR in the Capture-Fusion Assay Correlates With Clinical Response. Although TVR was poorly effective in G3 HCV, a subgroup of patients (~30%) did respond.²⁶ To establish whether the capture-fusion assay could identify TVR-sensitive G3 HCV, pretreatment, blinded sera were obtained from 8 patients with G3 HCV who had received TVR monotherapy in a clinical trial.²⁶ TVR sensitivity of each sample in the capture-fusion assay correlated with clinical response, whereas a biochemical phenotyping assay did not¹² (Fig. 7). Three samples with the greatest TVR sensitivity in the capture-fusion assay corresponded to patients who responded clinically to TVR. These results indicate that the assay may predict patient responses to TVR.

Discussion

We have developed a capture-fusion technique that allows drug sensitivity of over 80% of HCV viral strains to be assessed. We found that resistance to TVR can be reliably determined, clinical sensitivity to cyclophilin and protease inhibitors can be identified, and there is a strong correlation between TVR sensitivity in our assay and clinical response in patients with G3 HCV, unlike conventional phenotyping assays. Cell-fusion techniques have been employed to investigate extrahepatic HCV replication,³¹ but this is the first report using cell fusion to facilitate replication of patient-derived HCV strains.

Emerging therapies for chronic HCV infection involve combinations of DAAs. Different combinations have differential effects on different viral strains, for example, faldaprevir with a non-nucleoside NS5B inhibitor cures most patients with G1b HCV, but only

43% of patients with G1a.² Accurate phenotyping of infecting virus before antiviral therapy may be valuable in determining appropriate drug combinations in future therapeutic regimens. Although the number of drug combinations tested in our assay is relatively small, we have no reason to expect that the assay will not identify viral sensitivity to a wide range of different therapies.

We investigated compounds with clinical trial data that differ in different genotypes. We found a close correlation between activity in patients and our assay, suggesting that the capture-fusion model may be useful in assessing sensitivity to DAAs. For the protease inhibitor, TVR, our assay accurately identifies viruses with known resistant variants, and, of interest, we identified a single patient with WT protease sequence and a poor therapeutic response to TVR whose virus was phenotypically resistant to TVR. These data suggest that novel resistant variants to TVR may exist and lie outside the classical resistance-associated regions. Further work will be required to delineate this region and identify the frequency of these variants.

The clinical response to Peg-IFN and RBV therapy for HCV is dominated by host (interleukin [IL]28B)^{32,33} and viral genotype. In an assessment of viral IFN sensitivity in 8 patients with diverse responses, we noted reduced sensitivity in patients who showed no clinical response. The archival samples analyzed could not be correlated with IL28B genotype, and insufficient samples were available to assess RBV sensitivity. Further studies will be needed to investigate the complex interactions between host IL28B genotype, viral IFN sensitivity, and clinical outcome. We noted a reduced response to RBV in patients with G3 HCV who relapsed, when compared to patients who achieved SVR, suggesting that RBV sensitivity may be an important determinant of response in this genotype. Further studies, including viral sequencing and phenotyping in chimeric replicons, will be required to confirm this observation and are underway.

It has long been recognized that monocytes from patients with HCV contain viral fragments, but it is unclear whether these cells contain replicating virus. We find that fusing monocytes from patients with HCV to replication-permissive hepatocytes allows viral replication in chimeric cells, indicating that monocytes contain viable HCV. The role of sequestered virus in viral persistence and the emerging immune response remains to be determined.

Interestingly, given the antiviral action of IFN- γ , we found that IFN- γ prestimulation of THP-1 was required, in addition to PMA, to achieve viral replication after fusion of HCV-exposed THP-1 with

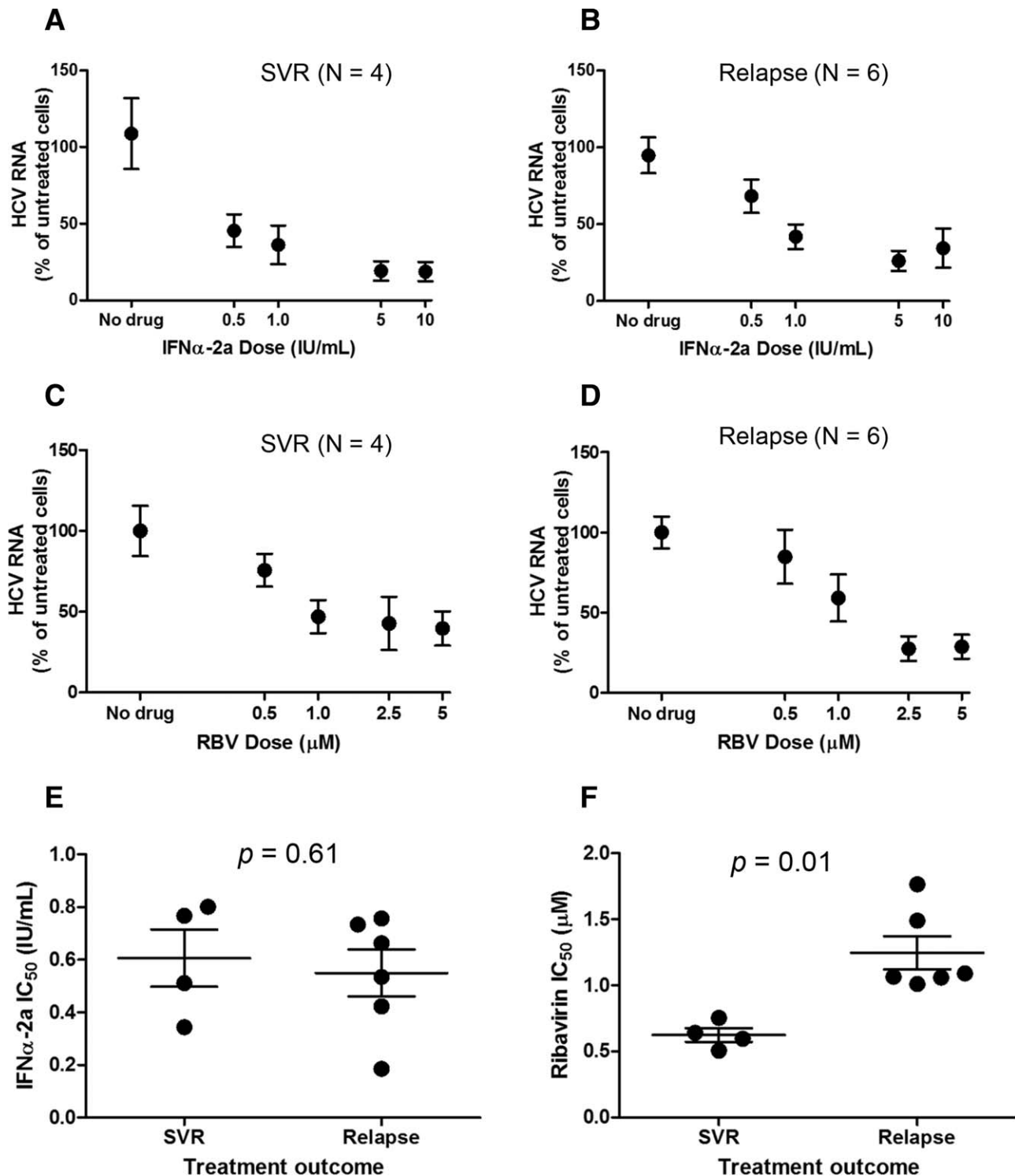


Fig. 6. Pre-treatment RBV sensitivity in the capture-fusion assay correlates with treatment response in G3 patients with cirrhosis treated with IFN/RBV. Using archived pretreatment sera from 10 G3 patients with cirrhosis who relapsed or achieved SVR after Peg-IFN and RBV treatment, no difference in IFN sensitivity was observed according to treatment outcome (A, B, and E). HCV strains from patients who achieved SVR were more sensitive to RBV in the capture-fusion assay than viral strains from patients who relapsed (C, D, and F). Graphs show mean \pm standard error of the mean.

Huh7.5. Others have observed Fc receptor up-regulation after PMA/IFN- γ treatment of monocyte cell lines, with enhanced uptake of HCV immune

complexes.²² Certainly, uptake of HCV into THP-1 cells appears independent of the classical HCV entry receptors, CD81, SR-B1, claudin, and occludin.

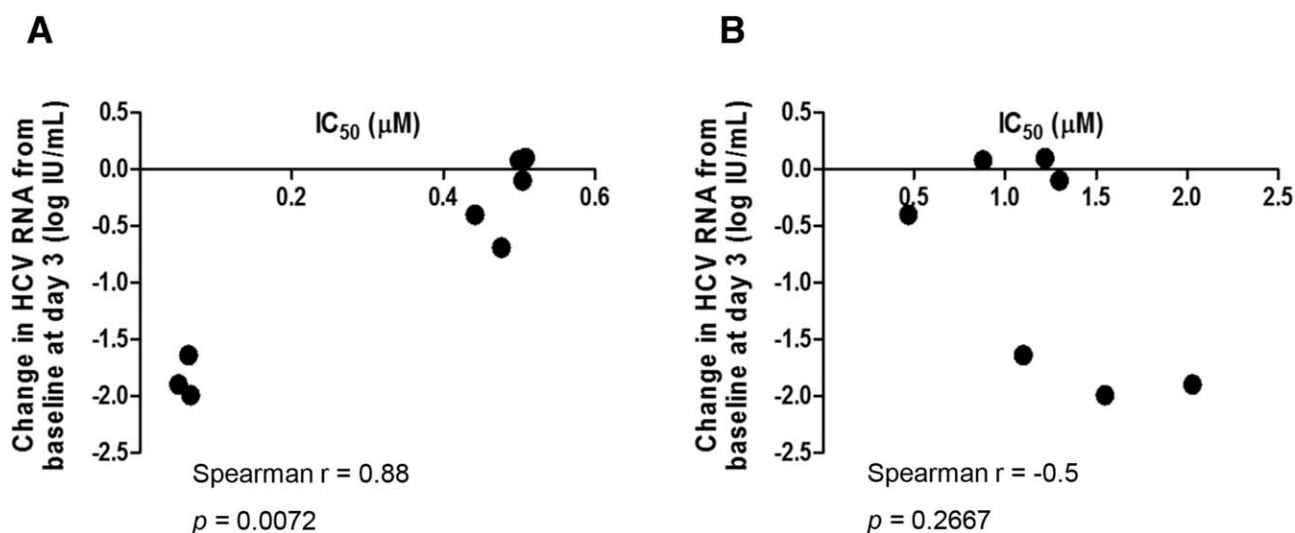


Fig. 7. Correlation between pretreatment TVR sensitivity and response to TVR in 8 patients with G3 HCV. Clinical response to TVR monotherapy is shown on the y-axes and could be predicted by the capture-fusion assay (A), but not by a biochemical phenotyping assay in which the viral NS3 was subcloned and enzymatic activity assessed in the presence and absence of TVR using a fluorescence resonance energy transfer cleavage assay¹² (B).

Significant replication of HCV does not occur in THP-1 cells, suggesting that fusion with Huh7.5 may provide access to permissive factors absent in THP-1. These may include the phosphoinositide kinases, PI3K or PI4K, given that the PI3K/PI4K inhibitor, wortmannin, reduced replication after fusion. Delivery of HCV to Huh7.5 cells by fusion bypassed MxA induction, despite retention of IFN-signaling pathways. This suggests that one mechanism underlying the capture-fusion process is delivery of virus to hepatocytes without an intracellular innate immune response. However, other factors are likely to play a role and elucidating the mechanisms underlying HCV replication after monocyte-hepatocyte fusion requires further work.

The assay described depends upon detection of low-level HCV RNA by PCR. Alternate techniques to detect HCV proteins directly (e.g., western blotting and IF) were attempted, but the level of viral protein was too low to allow detection (western blotting) or was masked by high levels of nonspecific background staining in fused cells (IF). PCR is a well-validated technique to detect viral replication, and the robust responses observed in the assay support the value of this approach.

In summary, we have developed a capture-fusion assay that permits replication of patient-derived HCV of all viral genotypes *in vitro* and enables testing of sensitivity to antiviral drugs. Genotype-specific responses to novel antiviral drugs and detection of resistant viral strains have been demonstrated. IFN-responsive or poorly responsive viral strains can be distinguished, and the assay can identify the subgroup of

patients with G3 HCV who respond to TVR. The capture-fusion assay represents a promising new technique that may help identify the most appropriate treatment strategy for patients with chronic HCV.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.27570/supinfo

Pan-genotypic anti-HCV activity of SB 9200 assessed in the ‘capture-fusion’ replication assay

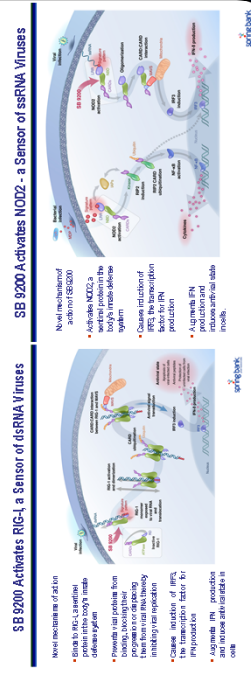
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Background and Aims

SB 9200 is a novel, first-in-class anti-HCV drug, which acts by enhancing the function of host cytosolic sensor proteins RIG-I and NOD2 that detect RNA viruses. SB 9200 has shown potent activity against HCV G1a and G1b replicons *in vitro* and is synergistic with other anti-HCV drugs such as telaprevir (NS3 protease inhibitor), NM283 (NS5B inhibitor), interferon and ribavirin. Since SB 9200 activates “host” targets instead of viral targets, it has the potential for pan-genotypic antiviral activity and a higher barrier to viral resistance.

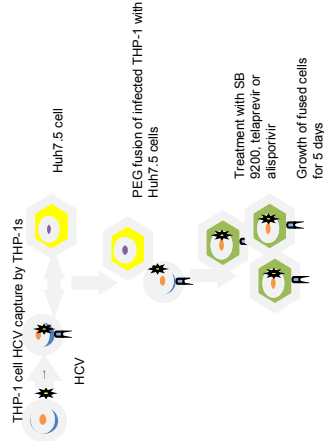
To explore the genotypic range of SB 9200, sensitivity of patient-derived HCV of different genotypes was tested in the recently developed capture-fusion assay, a novel HCV replication model that is highly predictive of clinical outcome of potential antivirals.



Methods

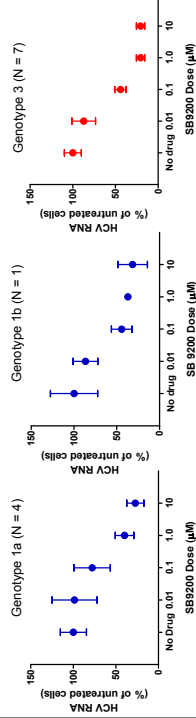
Pre-stimulated THP-1 cells were infected with serum from donors chronically infected with the common HCV genotypes G1 or G3, or from patients infected with the less prevalent G2, G4 or G6 HCV. The hybrid cells were treated once with a range of concentrations of SB 9200. For comparison, fused cells infected with G1 and G3 sera were treated with Telaprevir or Alisporivir.

The cells were cultured for 5 days, before quantification of HCV RNA by PCR. Dose-response curves were used to calculate IC₅₀ values for each experiment.

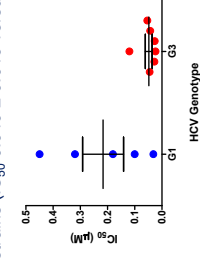


SB 9200 inhibited the replication of G1 and G3 HCV strains in the fused cells

Replication of HCV from sera of patients infected with G1 HCV and G3 HCV was inhibited by SB 9200 in a dose-dependent manner.

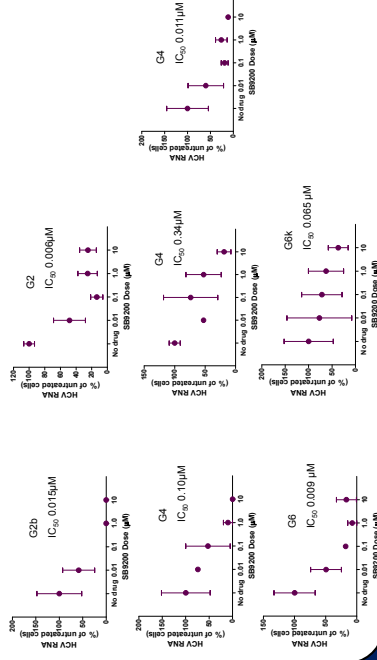


G3 isolates showed significantly greater sensitivity to SB 9200 than G1a/b strains (IC₅₀ 0.049 ± 0.013 versus 0.22 ± 0.076, *p* = 0.048).



SB 9200 inhibited the replication of G2, G4 and G6 HCV in fused cells

Sera from a small number of patients chronically infected with G2, G4 or G6 HCV also demonstrated sensitivity to SB 9200 in the capture-fusion model.

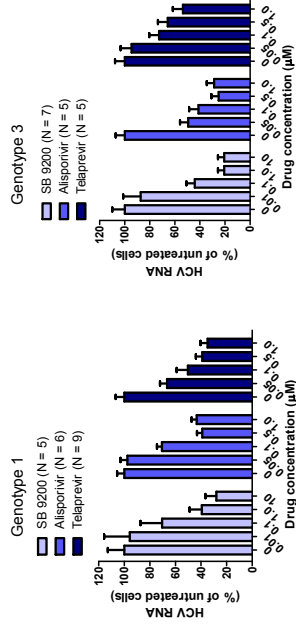


Sensitivity of both G1 and G3 HCV to SB 9200 compares favourably to Telaprevir and Alisporivir

For comparison, fused THP-1/Huh7.5 cells infected with sera from donors with G1 or G3 HCV were treated with a range of concentrations of alternate anti-HCV drugs, Telaprevir or Alisporivir.

G3 isolates were significantly more sensitive to SB 9200 than to Telaprevir (SB 9200 IC₅₀ 0.05 ± 0.01 μM, Telaprevir IC₅₀ 0.12 ± 0.03 μM, *p* = 0.01).

In G1 isolates, anti-viral potency of SB 9200 was comparable to that of Alisporivir in this replication model (SB 9200 IC₅₀ 0.22 ± 0.08 μM, Alisporivir IC₅₀ 0.14 ± 0.03 μM; *p* = 0.66). A similar pattern was seen in G3 isolates (SB 9200 IC₅₀ 0.05 ± 0.01 μM, Alisporivir IC₅₀ 0.04 ± 0.02 μM; *p* = 0.88).



Sensitivity of patient-derived G1 or G3 HCV to SB 9200, Alisporivir or Telaprevir in the capture-fusion assay. X-axes show concentration of each drug, y-axes show degree of inhibition of replication. Values are mean ± s.e.m.

Conclusions

These results demonstrate antiviral activity of SB 9200 against a diverse range of HCV genotypes *in vitro*. Of note, this compound shows very potent activity against patient-derived G3 isolates. These results support the potential use of SB 9200 as a pan-genotypic host-targeting anti-HCV agent that can be used with different classes of direct-acting antivirals in combination therapy.

Acknowledgements

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CD64 (FcγRI) is a novel receptor for HCV entry into monocytes

Morven E. Cunningham, Joseph Davidson-Wright, Meleri Jones, Joshua L. Wong, Jennifer A. Waters, Graham R. Foster

Background and aims

Monocytes from patients with HCV contain virus and we have shown that this virus replicates when monocytes are fused to the hepatoma cell line Huh7.5.

We have previously developed a replication system in which patient-derived HCV is "captured" by the monocytic cell line THP-1 and viral replication assessed after fusion of these cells to Huh7.5 cells.

Interferon-γ (IFN-γ) and PMA have previously been demonstrated to enhance association of HCV immune complexes with other monocytic cell lines¹.

Here we explored the receptors involved in monocyte capture/entry of patient-derived HCV, specifically those involved in hepatocyte HCV entry as well as Fcγ receptors (FcγR).

Methods

Unstimulated THP-1 or cells pre-stimulated with PMA and IFN-γ were incubated with sera from patients with chronic HCV and HCV RNA quantified by qPCR.

mRNA expression of classical HCV entry receptors and FcγR was compared in stimulated and unstimulated cells and surface receptor expression analysed by FACS.

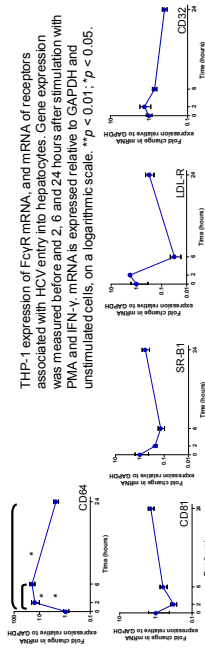
Stimulated THP-1 were incubated with blocking antibodies to candidate entry receptors prior to incubation with patient sera and PEG-mediated fusion with Huh7.5 cells.

HCV RNA was quantified immediately and up to 7 days after fusion.

Results are mean ± s.d. and p values were calculated using the Mann-Whitney U test.

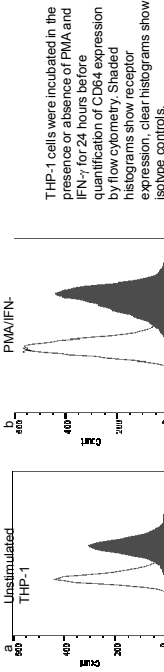
PMA/IFN-γ treatment of THP-1 cells increases CD64 gene expression

Incubation of THP-1 cells with PMA and IFN-γ led to increased expression of CD64 mRNA, but not that of CD32, CD81, SR-B1 or LDL-R. Other candidate receptors (claudin-1, occludin and CD16) were not expressed by THP-1 cells before or after stimulation.



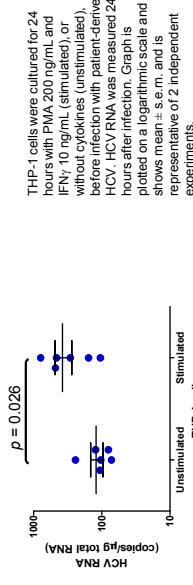
PMA/IFN-γ treatment of THP-1 cells increases CD64 expression at the cell surface

Cell surface expression of CD81, SR-B1 or CD32 was not affected by PMA/IFN-γ exposure.



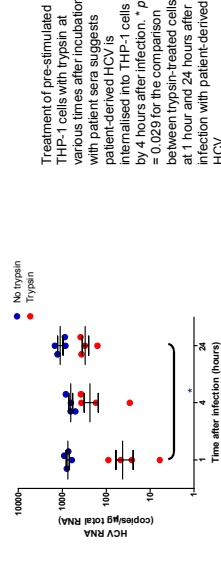
Pretreatment of THP-1 with PMA/IFN-γ enhances association of patient-derived HCV

Patient-derived HCV associated poorly with unstimulated THP-1 cells, but this was enhanced by 24 hours pre-stimulation with PMA and IFN-γ (121 ± 62 versus 380 ± 252 HCV copies/μg total RNA, p = 0.026)



Patient-derived HCV is internalised into pre-stimulated THP-1 cells

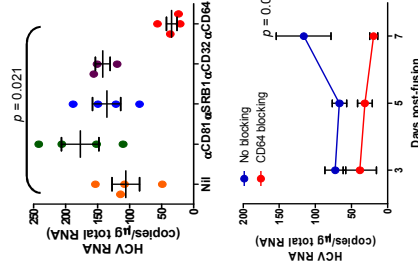
Trypsin treatment of stimulated THP-1 after exposure to serum from donors chronically infected with HCV confirmed internalisation of patient-derived virus. Internalisation occurred predominantly between 1 and 4 hours after infection.



Replication of patient-derived HCV after THP-1/Huh7.5 cell fusion was reduced by blocking CD64

Pre-incubation with CD64 blocking antibodies reduced the amount of HCV detectable immediately after fusion of infected THP-1 with Huh7.5 cells, and also reduced HCV replication in the fused cells (19 ± 12 versus 116 ± 100 HCV copies/μg total RNA 7 days after fusion, p = 0.005).

Blocking antibodies to CD81, SR-B1 or CD32 had no effect.



Conclusions

Uptake of patient-derived HCV into THP-1 monocytes is mediated primarily through CD64.

Blocking CD64 did not completely abrogate HCV uptake suggesting that other, as yet undefined receptors may also be involved but these are distinct from classical HCV entry receptors including CD81.

Although we found no evidence of HCV replication in THP-1 cells, replication occurred after fusion with Huh7.5 cells suggesting that HCV internalised into THP-1 via CD64 is replication-competent. This may have implications for viral persistence and relapse after antiviral therapy.

Reference

- Marino R, Delbis L, De Sanctis JB, Bianco NE, Toro F. Interaction of immune complexes isolated from hepatitis C virus-infected individuals with human cell lines. *Med Microbiol Immunol* 194; 73-80 (2005)

Acknowledgements

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Pre-treatment prediction of telaprevir response using a novel capture-fusion assay for HCV replication

Morven E. Cunningham, Alia Javaid, Jenny A. Waters, Joseph Davidson-Wright, Joshua L.C. Wong, Tanzina Haque, Malcolm Macartney, Geoffrey Dusheiko, William Rosenberg, Mike Jacobs and Graham R. Foster

Background and aims

Telaprevir enhances treatment response in genotype 1 (G1) HCV, but cure rates remain low amongst patients with prior treatment null response.

Genotype 3 (G3) HCV is insensitive to telaprevir, but a subset of G3 patients (~30%) may respond¹.

We have developed a novel capture-fusion assay to study patient-derived HCV.

Here we demonstrate that this assay can identify pre-treatment telaprevir sensitivity in patients with G1 and G3 HCV.

Methods

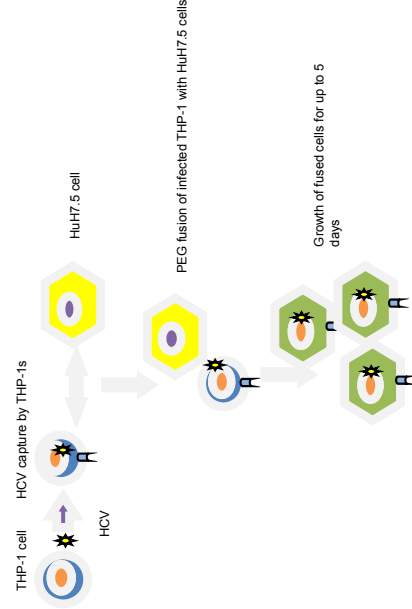
Pre-treatment and post-breakthrough samples were obtained from three G1 patients (previous 'null responders') who failed retreatment with telaprevir, pegIFN and ribavirin.

Pre-treatment serum samples were obtained from eight G3 patients treated with telaprevir monotherapy¹, blinded to virological response.

Pre-stimulated THP-1 cells were infected with donor serum and fused with HuH7.5 cells using polyethylene glycol.

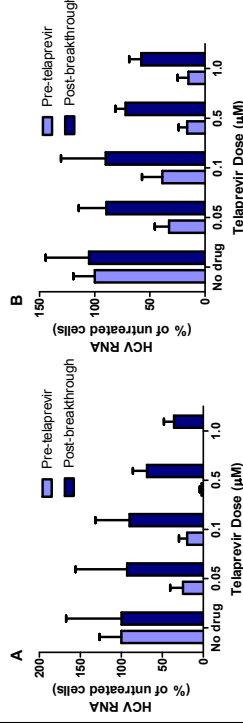
The fused cells were treated with telaprevir at a range of concentrations for 5 days.

After RNA extraction, of HCV RNA was quantified by real-time qPCR.



Loss of telaprevir sensitivity can be detected in the capture-fusion assay

Two G1 patients (A, B) responded to therapy with telaprevir, pegIFN and ribavirin then broke through (treatment week 12). Telaprevir sensitivity of pre-treatment and post-breakthrough serum samples was measured in the capture-fusion assay.



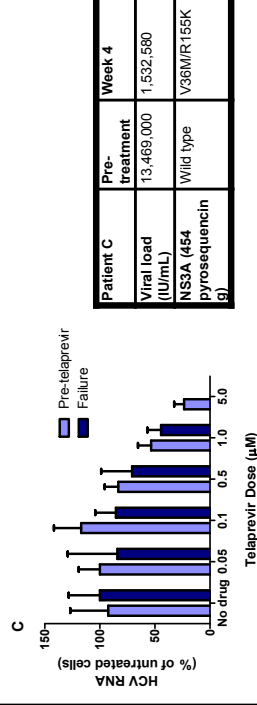
Patient A	Pre-treatment	Week 4	Week 12
Viral load (IU/mL)	2,068,670	129	1107
NS5A (454) pyrosequencing (g)	Wild type		V36M/R155K

Patient B	Pre-treatment	Week 4	Week 12
Viral load (IU/mL)	5,196,470	0	115,030
NS5A (454) pyrosequencing (g)	Wild type		V36M/R155K

Pre-treatment, both patients were sensitive to telaprevir (IC_{50} 0.041 μ M (A) and 0.052 μ M (B)). Telaprevir resistance was associated with loss of telaprevir sensitivity in the capture-fusion model (IC_{50} 0.743 μ M (A) and 0.610 μ M (B)).

Pre-existing telaprevir insensitivity can be predicted

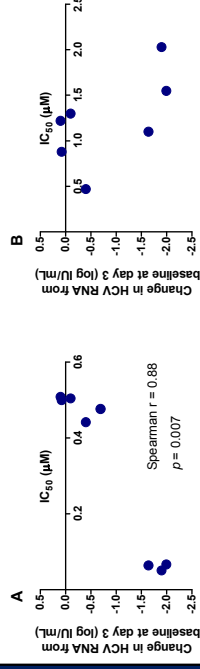
One G1 patient (C) treated with telaprevir, pegIFN and ribavirin reduced HCV RNA by <1 log₁₀ IU/mL at treatment week 4. Telaprevir sensitivity of pre-treatment and post-failure serum samples was measured in the capture-fusion assay.



Pre-treatment, patient C had an insensitive viral strain (IC_{50} 0.899 μ M). Acquisition of genotypic telaprevir resistance was associated with little further reduction in sensitivity (IC_{50} 1.66 μ M).

G3 patients who respond to telaprevir can be identified

Pre-treatment serum samples were obtained from eight G3 patients treated with telaprevir monotherapy¹, blinded to virological response. Telaprevir sensitivity was measured in the capture-fusion assay.



Pre-treatment telaprevir sensitivity correlated much better with response to telaprevir in the capture-fusion assay (A) than in a biochemical phenotyping assay (B) in patients with G3 HCV. X axes show telaprevir sensitivity (IC_{50}), y axes show clinical response to telaprevir monotherapy.

Conclusions

These data confirm the value of the capture-fusion assay in predicting individual patient telaprevir response.

A subset of genotype 1 patients with pre-existing telaprevir insensitivity, not readily detected by sequencing, can be identified.

Genotype 3 patients who respond to telaprevir can be distinguished from those who do not.

The capture-fusion technique may help identify the most appropriate treatment strategy with new antiviral agents.

References

1. Foster GR *et al.* Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. *Gastroenterology* 2011; 141(3): 881-889.

Acknowledgements

We are grateful to Janssen for providing G3 pre-treatment samples and results of biochemical phenotyping of these samples.

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Development and validation of a novel capture-fusion model to study primary HCV replication and anti-viral drug sensitivity

Morven E. Cunningham, Alia Javaid, Jenny A. Waters, Joseph Davidson-Wright, Joshua L.C. Wong, John McLauchlan and Graham R. Foster



Background and aims

HCV grows very poorly in culture, hampering development of novel antiviral agents

Drug development to-date has depended on a limited number of atypical strains which replicate *in vitro*

Previous work from our group has developed a fusion model for detection of viral replication in monocytes taken from patients with chronic HCV infection

Here we describe a novel replication assay using sera from subjects infected with different HCV genotypes and validate its use as a tool to screen for antiviral drug sensitivity

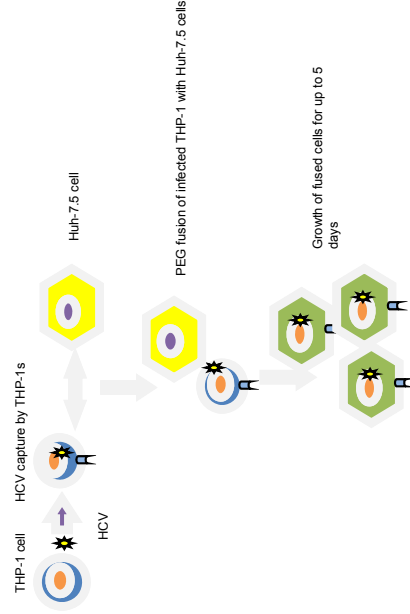
Methods

THP-1 cells were pre-stimulated with cytokines and infected with serum derived from patients with chronic HCV infection.

Infected THP-1 cells were fused with the human hepatoma cell line Huh-7.5, using polyethylene glycol (PEG).

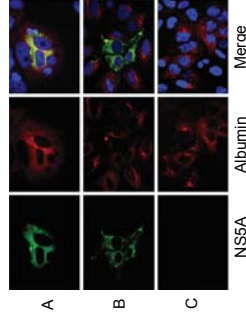
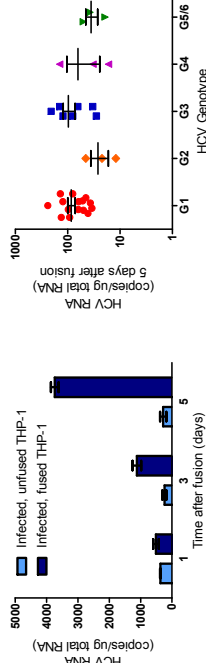
The fused cells were grown in culture for up to 5 days in the presence or absence of antiviral agents

After RNA extraction, of HCV RNA was quantified by real-time qPCR.



Assay development

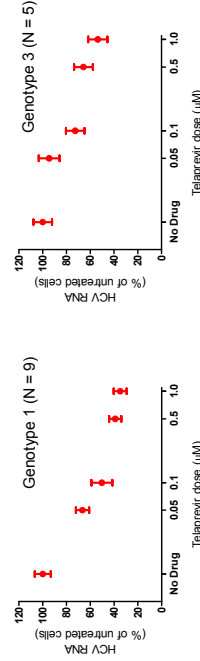
HCV replication occurs after fusion of infected THP-1 with Huh 7.5 cells. 5 days after fusion, HCV RNA is quantifiable from cells infected with all viral genotypes.



Detection of HCV protein production by immunofluorescence confirms co-staining of the HCV NS5A protein (green) in albumin-producing Huh 7.5 cells (red). Row A, prestimulated THP-1 infected with patient serum and fused with Huh 7.5. Row B, Huh 7.5 cells infected with JFH-1 (positive control); Row C, uninfected Huh 7.5 cells (negative control).

Assay validation: genotype specific telaprevir dose response

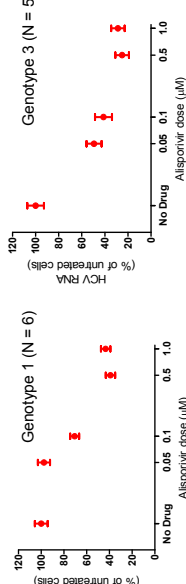
Telaprevir shows potent antiviral activity in patients with genotype 1, but not genotype 3 infection both in the clinic and in our assay



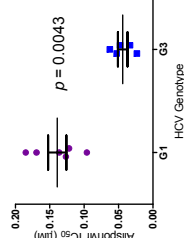
In separate capture-fusion experiments using sera from patients with genotype 1 (N = 9) or genotype 3 (N = 5) HCV, fused cells were treated with varying concentrations of telaprevir. Dose-response curves were constructed and used to calculate telaprevir IC₅₀ values for each patient.

Assay validation: genotype specific alisporivir dose response

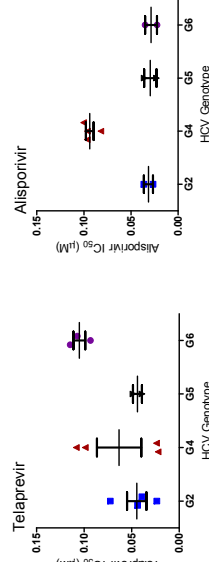
Alisporivir is more effective against genotype 3 than genotype 1 HCV both clinically and in our assay.



In separate capture-fusion experiments using sera from patients with genotype 1 (N=6) or genotype 3 (N=5) HCV, fused cells were treated with varying concentrations of alisporivir. Dose-response curves were constructed and used to calculate alisporivir IC₅₀ values for each patient.



Preliminary results indicate that telaprevir and alisporivir sensitivity of isolates of other viral genotypes can also be estimated



Conclusions

Replicating HCV from patients with diverse genotypes could be successfully transferred to Huh-7.5 cells using the monocytic 'capture-fusion' approach.

Replication was inhibited by the anti-HCV agents telaprevir and alisporivir, which correlated with genotype-specific drug response seen in clinical trials.

This *in vitro* assay may enable the tailored therapy of treatment strategies for HCV infected subjects receiving new direct acting antiviral agents.

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Financial disclosures: MEC, AJ, JW, JDW, JLCW and JM have nothing to disclose. GRF has received consultancy and speaker fees from Abbott, Boehringer-Ingelheim, BMS, Chugai, Janssen, Merck, Novartis and Roche.
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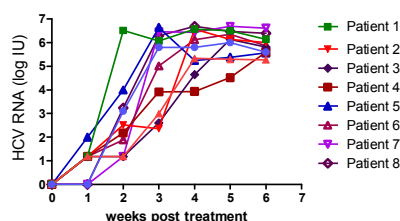
Presence of viable HCV RNA in monocytes at the end of treatment predicts relapse in genotype 3 HCV infection

Morven E. Cunningham, Alia Javaid, Jenny Waters and Graham R. Foster

Centre for Digestive Diseases, Blizard Institute, Barts and The London School of Medicine and Dentistry

1. Background and aims

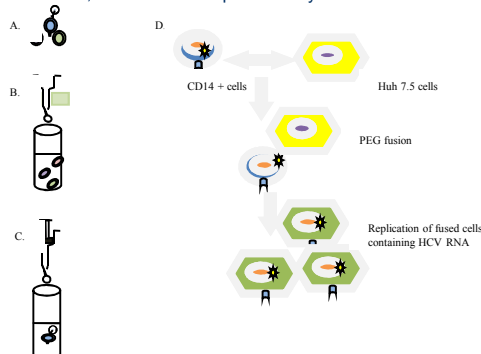
- Genotype 3 HCV is generally regarded as "easy to treat" based on clinical trial results showing response rates of up to 80%
- Response rates as low as 45% have been observed in real-world studies, particularly in patients with advanced fibrosis or cirrhosis
- Most patients who fail treatment for G3 HCV initially respond to antiviral therapy, but relapse after the end of treatment
- Data from our group indicates that relapse occurs rapidly after cessation of therapy, indicating viral persistence in a sanctuary site



- HCV RNA has been demonstrated in peripheral blood mononuclear cells (PBMCs) from patients with chronic HCV, but whether viral replication occurs in these cells remains controversial.
- We have recently developed a novel cell fusion technique to assay replication of patient-derived HCV
- This study uses our fusion assay to test the hypothesis that viable HCV in monocytes at the end of treatment predicts relapse in patients with genotype 3 HCV.

2. Methods

- PBMCs were isolated from whole blood taken from patients at the end of treatment for chronic genotype 3 HCV (A).
- CD14 (+) monocytes were isolated from total PBMCs by magnetic cell separation (B-C).
- CD14 (+) monocytes were fused with the human hepatoma cell line Huh-7.5, using polyethylene glycol (PEG) (D).
- The fused cells were grown in culture for up to 5 days, in the presence or absence of antiviral agents
- After RNA extraction, HCV RNA was quantified by PCR.



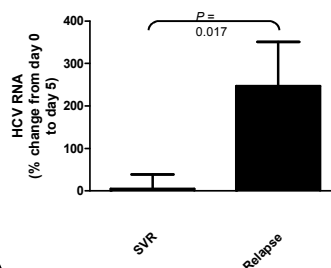
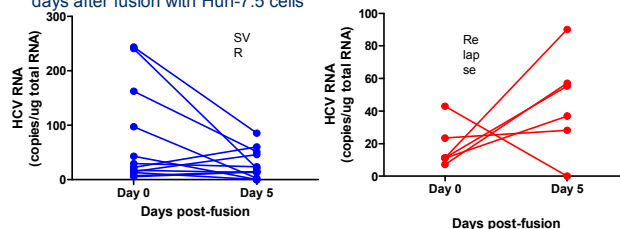
3. Results

18 sequential patients at the end of 24 weeks of pegylated interferon and ribavirin therapy for genotype 3 HCV were recruited into the study. Baseline characteristics and outcome of treatment were as shown.

Characteristic	N = 18
Gender (M:F)	6:12
Age, years (mean \pm SD)	43.8 \pm 10.3
Viral load (median, range)	2.24 $\times 10^5$ (0.12 $\times 10^5$ – 1.45 $\times 10^7$)
Fibrosis score (median, range)	3 (0 – 6)
Ethnicity: Caucasian	2
Bengali	1
Pakistani	15
Treatment outcome (SVR:Relapse)	17:6

Patients who relapsed were slightly older (mean age 52.3 \pm 8.0 versus 39.6 \pm 8.7 years, $P = 0.02$) and had slightly higher fibrosis scores (mean 3.8 \pm 1.9 versus 1.8 \pm 1.2, $P = 0.04$) than patients with SVR. Baseline viral load did not differ.

HCV RNA was quantified in unfused monocytes (Day 0), and in monocytes 5 days after fusion with Huh-7.5 cells



The amount of HCV RNA detectable in monocytes from most patients who relapsed increased after fusion, indicating viral replication in the fused cells. The opposite trend was seen in fused monocytes from patients with SVR. Overall, the percentage change in HCV RNA detectable 5 days after cell fusion was significantly greater in relapsers than patients with SVR.

4. Conclusions

- These data demonstrate that the presence of replication-competent HCV in monocytes at the end of treatment may predict relapse in patients with G3 HCV.
- Monocytes may act as a sanctuary site for HCV virions during interferon-based treatment, facilitating relapse after withdrawal of therapy.
- It may be possible to tailor the duration of therapy for patients with genotype 3 HCV infection, based on clearance of viable HCV from monocytes.
- Alternatively, novel direct-acting antiviral agents could be used as add-on therapy for patients with persistent viable HCV RNA in monocytes after 24 weeks of pegylated interferon and ribavirin therapy
- The value of either approach in reducing rates of relapse needs to be investigated in clinical trials.

Early experience with telaprevir for patients with advanced fibrosis or cirrhosis

Morven E. Cunningham, Josephine Schulz, Louise Payaniandy, Yiannis Kallis, Patrick Kennedy, Paul Kooner, Richard Marley and Graham R. Foster

The Royal London Hospital, Barts Health NHS Trust

1. Background

- The direct-acting HCV protease inhibitor telaprevir has recently been licensed for treatment of chronic genotype 1 HCV infection, and promises significant improvements in sustained virological response (SVR) for these patients.
- The patients who may benefit most from novel HCV therapies, namely those with advanced fibrosis or cirrhosis who have previously failed to respond to pegylated interferon (pegIFN) and ribavirin treatment, are relatively poorly represented in the telaprevir clinical trials.
- Results recently presented by the French early access programme (CUPIC) suggest that telaprevir has excellent antiviral efficacy but a high incidence of adverse events in cirrhotic patients with previous relapse or partial response to pegylated interferon and ribavirin (non-responders were not included).
- Efficacy, safety and tolerability were assessed in patients with genotype 1 HCV and advanced fibrosis/cirrhosis who have received telaprevir-containing treatment at the Royal London Hospital, including those with previous null response to pegylated interferon/ribavirin therapy

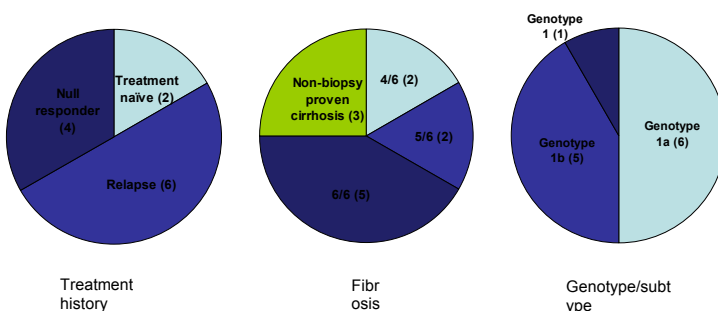
2. Methods

Laboratory results and case notes were reviewed for all patients treated with pegylated interferon, ribavirin and telaprevir at Barts Health NHS Trust between September 2011 and June 2012.

3. Patient Demographics

Twelve patients with genotype 1 HCV commenced treatment with pegylated interferon α -2a, ribavirin and telaprevir between September 2011 and June 2012.

Demographic	N = 12
Gender (M:F)	8:4
Age, years (median, range)	58.5 (35 – 66)
Baseline viral load (median, range)	3.06 $\times 10^6$ (6.8 $\times 10^3$ – 8.0 $\times 10^6$)



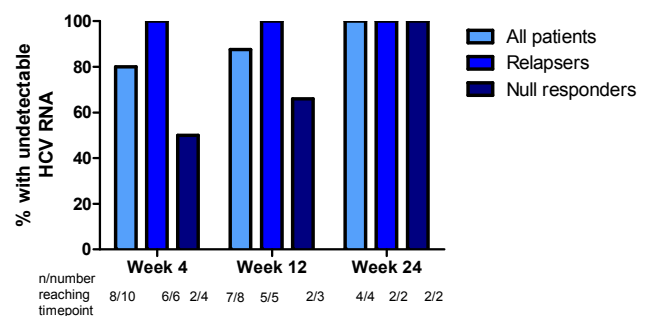
The majority of patients were male, with a relatively high viral load.

All patients had advanced fibrosis/cirrhosis either proven by biopsy (Ishak score 4-6), or with clinical, radiological and biochemical features suggestive of cirrhosis.

Most patients were treatment-experienced.

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4. Antiviral efficacy



Treatment efficacy is shown as the percentage of patients with undetectable HCV RNA who had reached the relevant timepoint.

2 patients (both prior null responders) had detectable HCV RNA at week 4. One patient stopped therapy, the other continued as HCV RNA was <1000 (41IU/mL). This patient had negative HCV RNA at week 8, but was positive at week 12 and stopped therapy. One patient withdrew prior to week 4 and one patient has not yet reached week 4.

One further patient withdrew from all therapy between weeks 4 and 12. 2 patients stopped telaprevir at week 10, but continued pegIFN and ribavirin and HCV RNA was undetectable at week 12.

5. Safety and tolerability

- 2 patients (17%) experienced serious adverse events during telaprevir treatment
- One patient required admission for blood transfusion
 - One patient required admission for treatment of leukocytoclastic vasculitis as well as blood transfusion

The second patient died at week 21 of treatment, with decompensated cirrhosis and E. coli sepsis.

2 patients (17%) stopped telaprevir therapy at week 10 due to adverse events; one due to leukocytoclastic vasculitis and the other due to moderate rash. Both continued pegIFN and ribavirin.

2 patients (17%) stopped all therapy at weeks 2 and 4 due to poor tolerability.

Other adverse effects are summarised below.

Adverse effect	Number affected (%)
Fatigue	12 (100)
Pruritis	4 (33)
Depression	4 (33)
Rash	3 (25)
Anal pain	3 (25)
Nausea	3 (25)
Gastrointestinal disturbance	3 (25)
Oral candidiasis	2 (17)
Anaemia (Hb <10 g/dL)	6 (50)
Transient hyperbilirubinaemia	3 (25)

4. Conclusions

- Telaprevir in combination with pegIFN and ribavirin appears efficacious in patients with advanced fibrosis or cirrhosis, who have previously failed treatment with pegIFN and ribavirin alone, including prior null responders.
- The incidence of significant side effects in this subgroup of patients is high and necessitates frequent follow-up with medical support.
- Side effects, particularly rash, may limit duration of telaprevir treatment. Whether this impacts on SVR remains to be seen.

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